特集　環境化学物質の作用メカニズムを解き明かす

重金属汚染による生物撹乱作用の分子基盤

Molecular Targets of Organotin Compounds in Endocrine Disruption

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重金属は生物に対し強い毒性を示すものが多いが，一般的にこのような低分子化合物の毒性は作用点が多岐に渡っており，分子レベルでの毒性発現機構の解明は困難であることが多い．一方で，近年の内分泌撹乱物質問題で話題となった有機スズ化合物は，貝類などの特定の生物種に特徴的な生殖毒性を誘引するが，最近，有機スズ化合物が核内受容体であるRXRやPPARγの強力なアゴニストとして作用することで，その毒性を発揮することが明らかとなってきた．本稿では，有機スズ化合物の核内受容体を介した生物撹乱作用について概説する．

key words
有機スズ，アロマターゼ，インポセックス，RXR，PPARγ，内分泌撹乱

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はじめに

重金属は生物に対し強い毒性を示すものが多く，鉱山や工場，産業廃棄物などから排出される重金属が，しばしば水源や土壌などの環境中に放出されてヒトの健康に影響を与えるなど，公害の原因となったりする．我々日本人にとって重金属毒性と言えば，おそらく多くの人がイタイイタイ病や水俣病といった公害を思い浮べるだろう．現在では，これら公害は過去に起こった歴史上の出来事であり，その作用機構の解明を含め，すでに解決した問題であるかのように認識されているかもしれない．しかしながら，低分子化合物の毒性発現には多くの分子や作用が関わっていると考えられ，その分子レベルでの作用機構解明は一般的に非常に困難である．イタイイタイ病や水俣病においても，これらの原因物質がカドミウムやメチル水銀であることは明らかとなっているが，その分子レベルでの毒性発現機構について解明されたとは言いがたいのが現状である．

一方で，近年話題となった内分泌撹乱物質（endocrine disrupting chemical；EDC）問題においても，船底塗料や漁網防汚剤などに使用されてきたトリブチルスズ（tributyltin；TBT）やトリフェニルスズ（triphenyltin；TPT）に代表される有機スズ化合物（図1）が，貝類に対してではあるものの，極低濃度で雌を雄性化し，繁殖不能にする状態（インポセックス）を誘導する[1)~3)]ことから，ヒトを含めた生物へのEDC作用が懸念されてきた．このような有機スズ化合物の貝類への影響は，EDC問題が提唱される以前から問題視されていたが，EDC問題が社会問題化してからは，有機スズ化合物の性ステロイドホルモンの受容体や合成経路に対する影響を中心に研究が行われてきた．これまでにも有機スズ化合物の毒性発現機構については様々な仮説が提唱されてきたが，有機スズ化合物においても他の重金属化合物と同様に，インポセックスの分子メカニズムやそれ以外の生物への毒性発現作用については不明な点が多く残されていた．しかしながら最近，これらの有機スズ化合物については，その毒性発現に関わる分子メカニズムが明確化しつつある．本稿では，有機スズ化合物の生物撹乱作用とその分子メカニズムについて，筆者らが最近得た知見を含めて紹介したい．

Ⅰ．有機スズ化合物のアロマターゼ阻害説

有機スズ化合物は，スズ原子にアルキル基やフェニル基が共有結合する構造を有し，官能基が1個結合するモノ体から4個結合するテトラ体まで多くの化合物から構成される人工化合物群である（図1）．これらの有機スズ化合物は，プラスチックの可塑剤や化学反応の触媒として各種化学工業で使用されるとともに，その殺生物能を利用して農薬や木材防腐剤，船底塗料，漁網防汚剤として使用されてきた．しかしながら，世界各地でこれらの化合物の水域汚染（主に海洋汚染）が顕在化し，また有機スズ化合物が貝類の雌に対してペニス様の突起を発生させるインポセックスを誘導することが明らかとなってからは，有機スズ化合物の水棲生態系へのみならず，汚染した海産物を食したヒトへの影響も問題視されるようになってきた．さらに有機スズ化合物は，元来毒性が強いことから，比較的古くから哺乳動物に対し

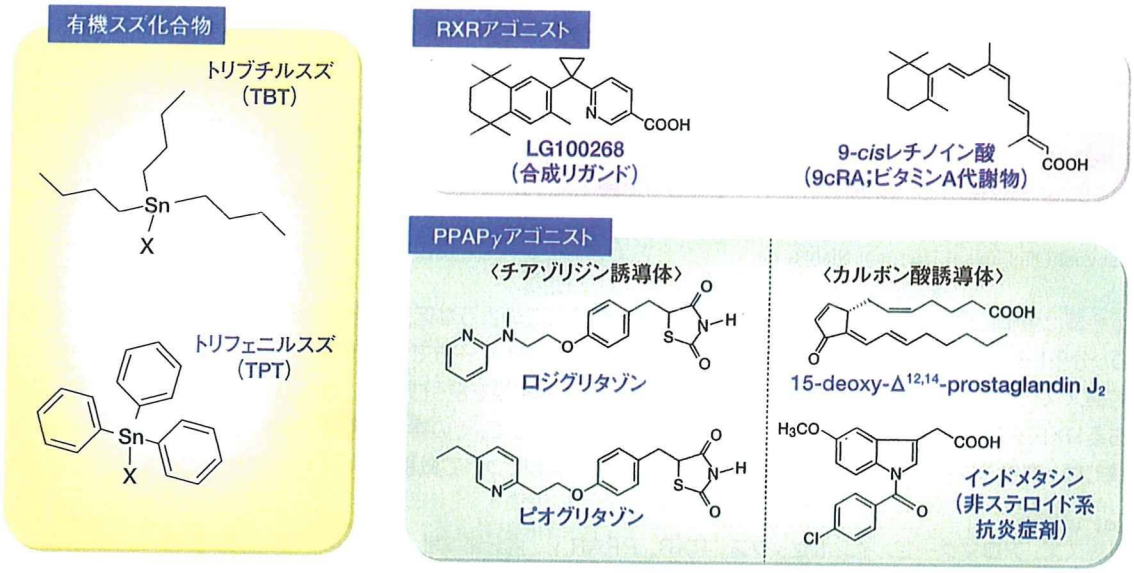


図1. RXR，PPAR γアゴニストと有機スズ化合物の化学構造

TBTやTPTなどの有機スズ化合物は，スズ原子（Sn）にアルキル基やフェニル基が共有結合する構造を有している．官能基が1個結合するモノ体から4個結合するテトラ体まで存在している．RXRアゴニストは，一般的に極性官能基と疎水性炭化水素基をリンカーで連結したような構造を有しているが，有機スズ化合物はそのような構造を有していない．またPPAR γアゴニストであるインスリン抵抗性糖尿病治療薬のようなチアゾリジンジオン構造も有していない．

て神経毒性や免疫毒性などを示すことが知られていた[4]が，その毒性発現機構についてはこれまでに統一した見解は得られていない．

一方で，有機スズ化合物の毒性発現には，いくつかのユニークな点が認められる．それは，①特定の生物種（貝類）に対して明確な生殖異常（インポセックス）を誘導する，②EDC問題においては，大部分の化合物が雌性化またはエストロゲン様作用が疑われているのに対し，雄性化作用が疑われている，③雄性化作用が疑われているが，エストロゲンレセプター（estrogen receptor：ER）やアンドロゲンレセプター（androgen receptor：AR）にはまったく親和性を示さない，④非特異的な細胞毒性を示す濃度よりも，かなり低濃度でインポセックスを誘導する，という点である．有機スズ化合物のインポセックスにおけるメカニズムについては様々な仮説が提唱されているが，②や③の観点から，有機スズ化合物はホルモンレセプターに直接作用するのではなく，ステロイドホルモン代謝に影響を与えることで誘導されるという説が有力視されていた[1), 2)]．

エストロゲンおよびアンドロゲンは，コレステロールを出発物質とし，モノオキシゲナーゼであるシトクロムP450と水酸基またはケト基の酸化または還元を触媒する脱水素酵素により生成されるが，有機スズ化合物はアンドロゲンからエストロゲンへの変換酵素であるアロマターゼの活性を阻害するのではないかと考えられた（図2）．すなわち，アロマターゼの酵素活性を阻害することで，体内のエストロゲン濃度の上昇を抑制し，アンドロゲンの濃度を上昇させる結果，雄性化を引き起こすのではないか？という“アロマターゼ阻害説”である[2)]．では，有機スズ化合物は本当にアロマターゼの活性を阻害するのであろうか？

CookeやHeidrichらのグループは，ヒトアロマターゼタンパク質を用いて，TBTがアロマターゼ活性を基質競合的に阻害することを報告している[5), 6)]．筆者らもヒト絨毛細胞株のミクロソーム分画を用いて，同様の検討を行ったが，確かにTBTおよびTPTともにアロマターゼの活性を阻害する[7)]．しかし，いずれの実験においても，その作用濃度は数μM～数十μMとかなり高濃度であり，通常の動物細胞は完全に死滅する濃度である[7)]．またこの他にも，高濃度のTBTおよびTPTが，アロマターゼ以外のステロイドホルモン合成関連酵素の活性を阻害するという報告が多数存在する[8), 9)]ことを考慮すると，有機スズ化合物のアロマターゼに対する酵素特異性についてはかなり疑問が残るところである．

インポセックスのアロマターゼ阻害説は，同じ水棲動物である魚類が，性ステロイドホルモンにより雌雄の表現型が決定されることに加え，貝類にTBTを投与すると体内のテストステロン濃度が上昇したり，またテストステロンやアロマターゼ阻害剤などで処理をするとインポセックスが誘導されるという結果に基づいている[1), 2)]．しかしながら，同じ水棲動物でもステロイドホルモンの生理的意義は，脊椎動物と無脊椎動物では大きく異なっていると考えられており，少なくとも無脊椎動物においては，古典的ステロイド

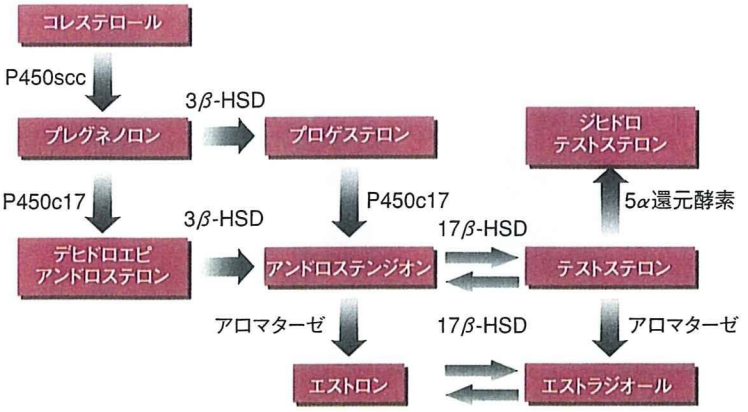


図2. コレステロールから性ステロイドホルモンへの合成経路

性ステロイドホルモンは,コレステロールを出発点としてモノオキシゲナーゼであるシトクロムP450と,水酸基またはケト基の酸化または還元を触媒する脱水素酵素により生成される.アロマターゼはアンドロゲンをエストロゲンに変換する律速酵素であることから,性ステロイドホルモンのバランスは主にアロマターゼにより保たれていると考えられる. HSD;hydroxysteroid dehydrogenase.

ホルモン（エストロゲン，アンドロゲン，プロゲステロン，糖質コルチコイド,鉱質コルチコイド)の受容体は存在しないと報告されている[10]. また貝類において,アロマターゼは同定されておらず，ERやARの存在が不確かな貝類に，果たして脊椎動物と同様の古典的ステロイドホルモンの合成および代謝経路が本当に存在するのか？ といった疑問もある.貝類のインポセックスにおいては,これまでに提唱された説の中で,“アロマターゼ阻害説”が圧倒的な支持を得てきた感がある.しかしながら,本説はこのような不確定な根拠の上に存在していることから，その信憑性についてはもう少し慎重に判断されるべきではないだろうか？

Ⅱ.ヒト胎盤のアロマターゼ発現に対する有機スズ化合物の影響

アロマターゼ活性が阻害されることで,体内の性ステロイドホルモンのバランスが崩れ,雌が雄性化するというインポセックスにおける“アロマターゼ阻害説”で想定されたような現象は，じつはヒトの発生段階においても認められる．ヒトの場合，妊娠期のエストロゲン産生（アンドロゲン代謝）の場は,卵巣から胎盤へと移行し,胎児-胎盤ユニットを形成することで,胎児および母体中の性ステロイドホルモンのバランスを維持している.そのホルモンバランスの維持において，特に重要な分子が胎盤のアロマターゼである．現に，胎盤のアロマターゼが欠損した女児においては,体内のアンドロゲン濃度が上昇し,ペニス様の突起が発生する仮性半陰陽(外性器は男性型であるが,内生殖器は女性型）となる[11]. このことは,性ステロイドホルモンのバランスや胎盤のアロマターゼが,ヒト胎児の生殖器官形成に非常に重要であることを示しており,化学物質が胎児に直接的な影響を与えなくとも,胎盤の内分泌機能を修飾することで胎児に少なからず影響を与える可能性を示唆している.

そこで筆者らは,有機スズ化合物をはじめとする様々な化学物質のヒト胎盤内分泌機能への影響を，非特異的な細胞毒性を示さない濃度域で検討を行った．その結果，TBTとTPTなどの有機スズ化合物が，濃度依存的にヒト絨毛細胞株のアロマターゼ活性とそのmRNAの発現を上昇させることが確認された[7),12)]．さらにこれらの有機スズ化合物は，ヒト胎盤においてエストロンを活性型エストロゲン（エストラジオール）に変換する酵素（17β-hydroxysteroid dehydrogenase type Ⅰ；17β-HSD Ⅰ）をも活性化し，エストラジオール産生を促進することも確認された[7),13)]．これらの結果は，これまでに貝類で予測されていたものとはまったく正反対であり，第Ⅰ章で述べた“アロマターゼ阻害説”に対する疑問を支持する結果であると言える.さらにEDC問題において有機スズ化合物は,雄性化を引き起こす典型的な化学物質であると認識されていたが,少なくともヒト胎盤に対してはエストロゲン産生を亢進することから,すべての生物種に対し雄性化作用を示すわけではないことが明らかとなった.このことは,有機スズ化合物がアロマターゼ以外の分子を標的として毒性を誘発する可能性を示唆している.

Ⅲ.核内受容体アゴニストとしての有機スズ化合物とアロマターゼ発現

ステロイドホルモン受容体をはじめとする核内受容体は，リガンド誘導性転写調節因子であり，ステロイドホルモンなどの脂溶性リガンドの情報を受け，特定の標的遺伝子群の発現を転写レベルで制御する．またこれらの内因性脂溶性リガンドは，それぞれの産生および代謝を互いに制御し合うことによって，生殖系や内分泌系などのホメオスタシスを維持していると考えられる．したがって，化学物質がERやAR以外の核内受容体に作用した場合においても，そのホメオスタシスが破綻する可能性は十分に考えられる．このような観点から,筆者らは,転写共役活性化因子と大腸菌アルカリホスファターゼとの融合タンパク質，および各種核内受容体のリガンド結合領域（ligand binding domain；LBD）を含むタンパク質とを利用した無細胞系リガンドスクリーニング法（CoA-BAP法；図3）[14)]を用いて，EDC作用が疑われている40種類の化学物質についてリガンドスクリーニングを行った[15)]．その結果，TBTおよびTPTが，ビタミンAの代謝物（9-*cis*レチノイン酸；9cRA）をアゴニス

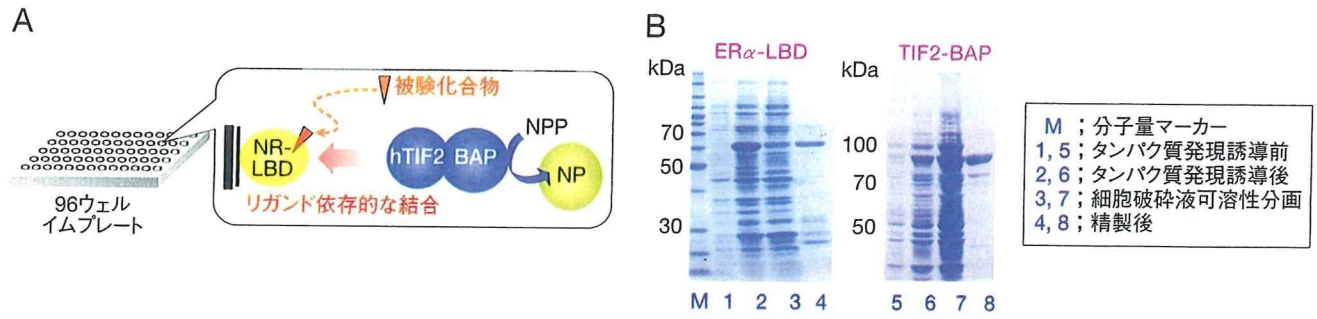
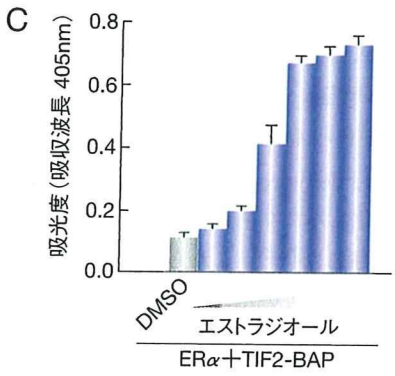


図3. CoA-BAP 法[14]



A：CoA-BAP（coactivator-bacterial alkaline phosphatase）法の概念図．核内受容体のリガンド結合領域（LBD）にアゴニストが結合したときに，コアクチベーターが結合できるようになる性質を利用している．精製したGST（glutathione-*S*-transferase）と核内受容体（NR）のLBDの融合タンパク質（NR-LBD）をGSH（glutathione）で固相化したマイクロプレートのウェルに，コアクチベーターであるTIF2とBAP（bacterial alkaline phosphatase）の融合タンパク質（TIF2-BAP）および被験化合物を加え，一定時間反応させる．被験化合物が核内受容体アゴニストとして機能する場合には，TIF2がNR-LBDに結合し，TIF2とBAPの融合タンパク質がウェル内に留まる．反応後，未反応の融合タンパク質を除き，BAPの基質であるNPP（*p*-nitrophenyl phosphate）を加える．NPPは無色であるが，BAPによって黄色の*p*-nitrophenolへと変換される．被験化合物の核内受容体に対する親和性が強いほどウェルに留まるTIF2とBAPの融合タンパク質は増加するため，*p*-nitrophenolによる発色の程度によって，被験化合物の核内受容体に対するアゴニスト活性を評価することができる．
B：ER αに対する評価系を作成した際に用いた精製タンパク質の電気泳動像．
C：構築したER αに対する評価系におけるエストラジオールの反応性．

トとするRXR（retinoid X receptor）と，インスリン抵抗性糖尿病治療薬であるチアゾリジン誘導体をアゴニストとするPPAR γ（peroxisome proliferator-activated receptor γ）のアゴニストとしての作用を有する可能性を見いだした[15]．これらの知見をもとに，ヒトやマウスの細胞を用いた検討を行ったところ，TBTとTPTは各受容体を介する転写を活性化し，そのEC$_{50}$は10～20nMであった[12), 15)]．また，精製タンパク質を用いた各受容体のLBDに対する解離定数の検討においては，RXR α，β，γに対しては既知のアゴニストである9cRAの各々約5，15，11倍[12)]，PPAR γに対してはチアゾリジン誘導体であるロジグリタゾンとほぼ同等であった（中西ら；未発表データ）．既知のPPAR γやRXRアゴニストとは構造がまったく異なるTBTやTPTが，これほどまでに強力なアゴニスト活性を示すのは驚きである（図1）．さらにTBTやTPT以外の有機スズ化合物についても検討を行ったところ，スズ原子に結合している官能基の構造や官能基の数によって，各受容体に対する転写活性化能や親和性が変動し，そのアゴニスト活性には明確な構造相関が認められた[12)]．

有機スズ化合物のこのようなアゴニスト活性が与える細胞機能への影響についても検討を行った．PPAR γとRXRは互いにヘテロ二量体を形成し，各々のアゴニスト依存的に転写を活性化することで，支配遺伝子の発現や脂肪細胞分化を誘導するが，TBTとTPTは前述の結果を反映して，マウスの脂肪細胞分化をPPAR γ/RXRの支配遺伝子の発現上昇を伴って誘導することも確認された[15)]．では，ヒト胎盤に対する影響はどうであろうか？ ヒト絨毛細胞株に，PPAR γアゴニストを添加しても，アロマターゼのmRNA発現や胎盤型のアロマターゼプロモーターの転写活性には影響は認められない[12)]．このことは，ヒト胎盤のアロマターゼの発現にはPPAR γ/RXRは関わっていないことを示唆している．しかしながら，RXR特異的アゴニストを添加した場合には，ヒト絨毛細胞株のアロマターゼ発現が上昇するうえ，胎盤型のアロマターゼプロモーターの活性も上昇する[12)]．RXRは，PPAR γ以外にもPPAR α，PPAR β，FXR（farnesoid X receptor），LXR（liver X recepter）ともヘテロ二量体を形成したり，またRXR自身でホモ二量体も形成する．これらの二量体も，PPAR γ/RXRと同様にRXRアゴニストで転写が活性化されるが，胎盤のアロマターゼ発現には前述のヘテロ二量体は関与していない[12)]．したがって，RXRアゴニストによるヒト胎盤でのアロマターゼ発現誘導は，RXRホモ二量体を介した作用であると考えられる．このことは，有機スズ化合物によるアロマターゼの発現誘導においても同様であり，有機スズ化合物はRXRホモ二量体を介して，ヒト胎盤のアロマターゼ発現を誘導するものと考えられる（図4）[12)]．

Ⅳ．核内受容体を介した有機スズ化合物の毒性

冒頭にも述べたとおり，有機スズ化合物は前述のEDC作用以外にも様々な毒性を誘導することが報告されているが，

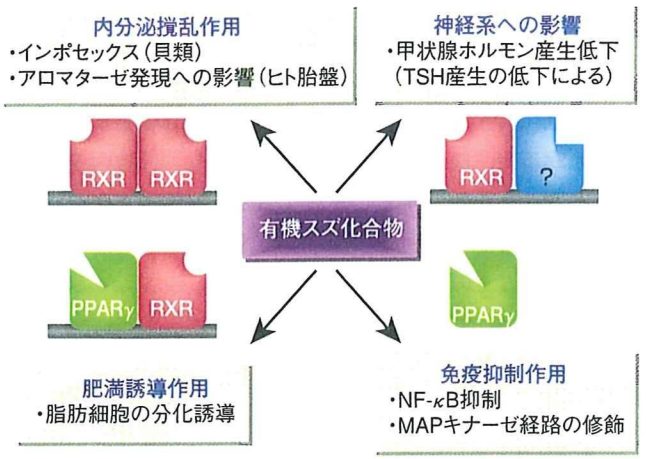


図4. 核内受容体を介した有機スズ化合物の様々な生物撹乱作用

有機スズ化合物はPPARγやRXRを介することで，これらの受容体を有する動物に様々な影響を与える可能性がある．

これらの毒性発現においてもPPARγやRXRが関わっている可能性がある（図4）．TBTは，ラットにおいて甲状腺刺激ホルモン（thyroid stimulating hormone；TSH）の産生低下を伴う甲状腺機能低下症を引き起こすことが報告されている[4]が，その一方で，RXR特異的アゴニストがマウスやラットにおいて同様の作用を引き起こす[16]ことから，TBTによるTSHや甲状腺ホルモンの産生低下はRXRを介して誘導されている可能性が考えられる．また有機スズ化合物は，免疫抑制作用やアレルギー反応の誘導などの免疫毒性も有する[4]が，PPARγが転写制御因子であるNF-κB（nuclear factor-κB）の転写活性を抑制したり，MAPキナーゼ（maitogen-activated protein kinase）経路によるシグナル伝達を修飾したりすることで，免疫担当細胞のサイトカイン産生などに影響を与えることが報告されている[17]ことから，有機スズ化合物による免疫毒性においてはPPARγが関わっている可能性が考えられる．さらに有機スズ化合物は，前述のとおり，PPARγ/RXRを介してマウスの脂肪細胞分化を誘導する[15]が，妊娠マウスにTBTを投与すると胎仔の肝臓などで脂肪組織の過形成が認められることも報告されていることから，最近では肥満誘導因子として作用する可能性も指摘されている[18]．これらの知見は，有機スズ化合物がPPARγやRXRを介することで，これらの受容体を有する動物に様々な影響を与える可能性があることを示唆している．

Ⅴ．インポセックスとRXR

RXRは，核内受容体ファミリーの中でも例外的に種を超えて保存されている受容体であり，昆虫類などの下等無脊椎動物においてもUSP（ultraspiracle）と呼ばれるオルソログが存在する．しかしながら，これら受容体のLBDは動物種によって大きく異なり，哺乳動物の内因性アゴニストとされている9cRAなどに対してUSPは応答しない．その一方で，最近イボニシなどの貝類においては，脊椎動物のRXR-LBDと相同性の高いRXRが存在することが報告された（図5）[19), 20)]．これらの貝類のRXRは，ヒトのRXRと同じく9cRAや有機スズ化合物と高い結合能を有する．また興味深いことに，これらの貝類に9cRAを投与すると，有機スズ化合物と同様にインポセックスが誘導されることから（図5），貝類のインポセックスはRXRを介して誘導されている可能性が示唆された（図4）[19), 20)]．有機スズ化合物を妊娠マウスなどに投与しても，インポセックスのような表現型は認め

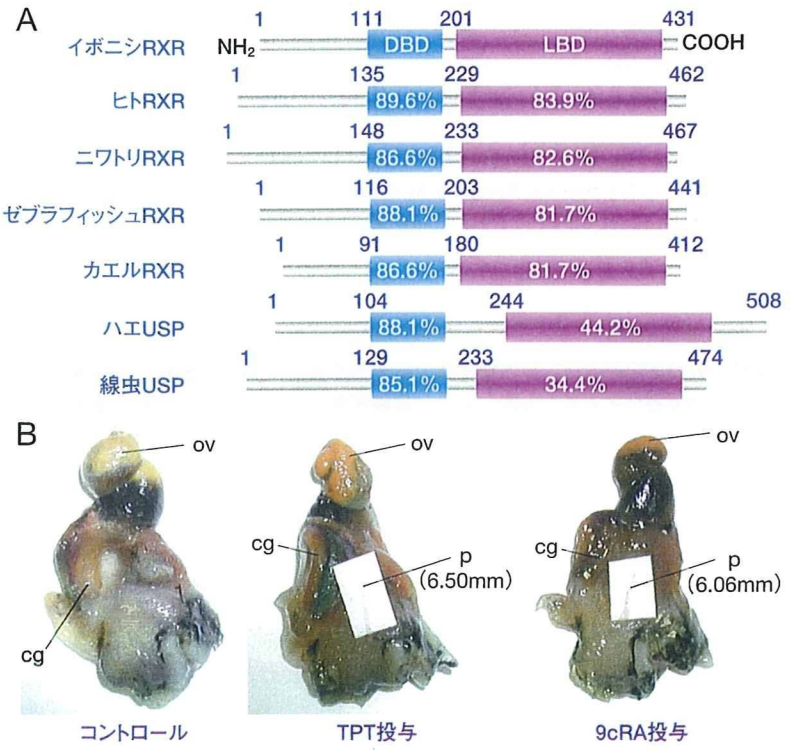


図5. 貝類のRXRと9cRAによるインポセックスの誘導

A：様々な生物種におけるRXR（USP）の構造の比較．すべての生物種においてDNA結合領域（DBD）の相同性は高いが，LBDの相同性はRXRとUSPでは大きく異なる．

B：9cRAとTPTによるイボニシのインポセックスの写真．各被験物質（1 μg/g wet wt.）をそれぞれ足部に注射して，人工海水中で1カ月間飼育後，取り上げて解剖した．ペニス部分には，ペニスを際立たせるために白い紙をのせている．cg；capsule gland（卵嚢腺），ov；ovary（卵巣），p；penis（ペニス：数値はペニス長）．Nishikawa J, et al: Environ Sci Technol (2004) 38: 6271-6276を一部改変．

写真提供：国立環境研究所 堀口敏宏先生

られないが，おそらくそれは，生殖器官形成などにおけるRXRの生理的意義が，生物種によって大きく異なるからであると考えられる．

おわりに

本稿では，有機スズ化合物の貝類とヒトアロマターゼへの影響を中心に，その分子メカニズムについて概説した．有機スズ化合物は，EDC作用が疑われている化学物質の中でも，きわめて低濃度で明確なEDC作用を誘導するが，それは核内受容体アゴニストとして作用することに起因することが明らかとなった．本来アゴニストとなることを意図されていない合成化学物質が，このように生理的アゴニストや合成アゴニストに匹敵するような影響を示す例はきわめてまれである．有機スズ化合物は，生物種によって誘発する表現型は異なるものの，様々な生物種に対してRXRまたはPPARγを介した毒性を引き起こす可能性が考えられる．今後は，PPARγやRXRなどの核内受容体に対する化学物質の作用や，様々な生物種におけるこれらの核内受容体の生理的意義を解明することによって，有機スズ化合物の生物撹乱作用がより明確になることを期待したい．

謝辞 本稿で紹介した成果は，大阪大学大学院薬学研究科 田中慶一先生（現・大阪大谷大学薬学部）のご指導とご助言により成し遂げられた成果であり，ここに深謝致します．また，研究を遂行してくれた廣森洋平君をはじめとする多くの学生に深謝致します．本稿を執筆するにあたり，イボニシの写真のご提供を賜りました国立環境研究所 堀口敏宏先生に深謝致します．

文献

1) Matthiessen P, et al: Environ Toxicol Chem (1998) 17: 37-43
2) Bettin C, et al: Helgol Meeresunters (1996) 50: 299-317
3) Horiguchi T, et al: Environ Pollut (1997) 95: 85-91
4) Benya TJ: Drug Metab Rev (1997) 29: 1189-1284
5) Cooke GM: Toxicol Lett (2002) 126: 121-130
6) Heidrich DD, et al: Steroids (2001) 66: 763-769
7) Nakanishi T, et al: J Clin Endocrinol Metab (2002) 87: 2830-2837
8) Doering DD, et al: Steroids (2002) 67: 859-867
9) Lo S, et al: J Steroid Biochem Mol Biol (2003) 84: 569-576
10) Escriva H, et al: Proc Natl Acad Sci USA (1997) 94: 6803-6808
11) Shozu M, et al: J Clin Endocrinol Metab (1991) 72: 560-566
12) Nakanishi T, et al: Mol Endocrinol (2005) 19: 2502-2516
13) Nakanishi T, et al: Biochem Pharmacol (2006) 71: 1349-1357
14) Kanayama T, et al: J Biochem (Tokyo) (2003) 133: 791-797
15) Kanayama T, et al: Mol Pharmacol (2005) 67: 766-774
16) Macchia PE, et al: Am J Physiol Endocrinol Metab (2002) 283: E326-331
17) Zhang X, et al: Int Immunopharmacol (2002) 2: 1029-1044
18) Grun F, et al: Mol Endocrinol (2006) 20: 2141-2155
19) Nishikawa J, et al: Environ Sci Technol (2004) 38: 6271-6276
20) Castro LF, et al: Aquat Toxicol (2007) 85: 57-66

for beginners

本稿では誌面の都合上，核内受容体の転写調節機能に関する基本的な事項の説明については割愛したが，以下の総説を参考にされたい．
・「核内受容体と創薬」山岡一良ら：実験医学（増刊）24: 152-160 (2006)

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β-Cryptoxanthin, a novel natural RAR ligand, induces ATP-binding cassette transporters in macrophages

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ABSTRACT

Despite its serious adverse effects, recent accumulating evidence suggests that a physiological retinoic acid receptor (RAR) agonist, all-*trans* retinoic acid (atRA), exhibits preventive effects on atherogenesis. Therefore, the present study was designed to explore novel natural RAR ligands with anti-atherogenic effects in order to identify and develop a drug without severe side effects. Among xanthophylls and carotenoids studied, β-cryptoxanthin and lutein exhibited RAR ligand activity in yeast two-hybrid system that was found to be completely abolished by the RAR pan-antagonist LE540. Furthermore, these molecules can bind the RAR ligand-binding domain in the CoA-BAP system but not RXR ligand-binding domain. These results indicate that both β-cryptoxanthin and lutein serve as ligands for RAR, but not RXR, although their binding affinity was three orders of magnitude lower than that of atRA. Additionally, when applied to macrophages, β-cryptoxanthin indeed was found to induce the ATP-binding cassette transporter A1 (ABCA1) and ABCG1 mRNAs, which exert anti-atherosclerotic effects by preventing cholesteryl ester accumulation in macrophages. The induction of ABCA1 proteins by β-cryptoxanthin as well as atRA was abrogated by LE540. In summary, β-cryptoxanthin appears to be more an efficient provitamin A source than other carotenoids and xanthophylls including β-carotene, since β-cryptoxanthin can act not only as a RAR agonist but also a source of vitamin A. Taking into account that the pharmacodynamics difference between β-cryptoxanthin and atRA, β-cryptoxanthin appears to exert beneficial effects on atherogenesis through RAR activation in the manner different from atRA.

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1. Introduction

Prospective cohort studies show a direct inverse association between a higher intake of fruits and vegetables and the development of cardiovascular diseases, such as coronary heart disease and stroke [1–4]. Among major carotenoids and xanthophylls, such as β-carotene, lycopene, α-carotene, lutein, and β-cryptoxanthin, β-carotene has been studied most extensively, but no benefits have been found for it in randomized trials [5,6]. These studies indicate that the apparent benefits from fruit and vegetable intake are not due to β-carotene itself and may be more properly ascribed to other nutrients or other factors present in these foods.

Atherosclerosis is a complex disease characterized by chronic inflammation [7] and by abnormal and excessive proliferation of vascular smooth muscle cells (VSMCs) [8]. In addition, the presence of activated macrophages and T cells in lesions caused by the disease suggest that cell-mediated immune reactions also occur during the development of atherosclerosis [9]. All-*trans* retinoic acid (atRA), a bioactive retinoic acid produced from vitamin A (retinol), is known to inhibit neointimal formation following balloon withdrawal injury of rat [10–12] or rabbit carotid artery [13]. atRA is also capable of inhibiting VSMC proliferation and promoting VSMC differentiation [14–16]. In regards to macrophages that are intimately involved in atherosclerosis development, atRA has been shown to induce ATP-binding cassette transporter A1 (ABCA1) [17] and a synthetic retinoid (AM80) reduced scavenger receptor expression [18], suggesting that atRA or its derivatives prevent foam cell formation by decreasing intracellular cholesteryl ester accumulation [19]. In addition, it was recently been reported that atRA exerts direct effects on T cells by suppressing T helper (T_h)1 development and enhancing T_h2 development that is essential for antibody responses [20,21]. Since the T_h1-driven immune response has been consistently shown to promote atherosclerosis, immunomodulation through T_h2 should be suitable to help reduce the progression of atherosclerosis. In total, these findings imply that atRA may be capable of attenuating the development of atherosclerosis by suppressing cellular immunity and stimulating humoral immunity.

To date, no clinically proven therapy exists for the successful management of neointimal formation in atherosclerosis or restenosis following angioplasty. However, atRA therapy appears to be a promising attractive approach for the treatment of neointimal formation especially given the clinical applications of atRA have been shown to have some success in the treatment of human diseases such as cancer, psoriasis, and leukemia. However, the therapeutic use of atRA has been excluded from consideration because of serious adverse effects such as skin or liver toxicity, teratogenesis, hypertriglyceridemia and development of resistance to atRA in addition to atRA syndrome characterized by fever, respiratory distress, interstitial pulmonary infiltrates, pleural and pericardial effusion, episodic hypotension, and acute renal failure [22,23].

Retinoid signaling is transduced by two families of nuclear receptors, the retinoic acid receptor (RAR), and the retinoid X receptor (RXR) [24,25]. These receptors belong to the superfamily of nuclear hormone receptors that act as ligand-activated transcription factors. RAR forms a heterodimer with RXR and the ligand–receptor complexes act as inducible transcription regulators of several genes by binding to specific retinoic acid response element. The pleiotropic effects of retinoids are primarily brought about by the existence of two families receptors (RAR and RXR), the RAR isotypes (α, β, and γ), and the RXR isotypes (α, β, and γ) in addition to the cross-talk of these receptors with other signaling pathways and the existence of multiple putative coactivators and corepressors [26]. In addition, since the RAR ligand-binding domain is capable of adopting a conformation exhibiting a large hydrophobic cavity similar to that observed in PPARγ-ligand-binding domain [27], various RAR ligands can induce various conformational changes in RAR, resulting in versatile gene expression and repression via the recruitment of different coactivators or corepressors as PPARγ ligands [28,29]. Furthermore, metabolic enzymes and retinoid binding proteins involved in storage, transport, uptake and sequestration of retinoids play critical roles in determining the availability of bioactive retinoids in cells [30]. This notion may indicate that RAR ligands that are metabolically different from the natural ligands for the RAR, such as atRA, 9-*cis*-RA, and 13-*cis*-RA [31], appear to be useful in treating various diseases.

Consequently, it seems to be very important to explore various RAR ligands that can create beneficial responses in a living body in order to develop therapeutic agents for cancer, atherosclerosis, and rheumatoid arthritis while simultaneously clarifying the signaling pathways associated with RAR. The present study was designed to search for natural RAR ligands among carotenoids and xanthophylls in order to develop potential therapeutic agents for cardiovascular diseases and to clarify the benefits of fruit and vegetable intake to prevent such diseases. That research had culminated in the identification of β-cryptoxanthin as a novel natural RAR agonist and a better provitamin A to activate RAR/RXR heterodimer than β-carotene.

2. Materials and methods

2.1. Chemicals and reagents

β-Cryptoxanthin, zeaxanthin, β-carotene, lycopene, all-*trans* retinoic acid, and 9-*cis* retinoic acid were obtained from Yashima Pure Chemical Co. Ltd. (Tokushima, Japan), Extrasyntheses (Genay, France), Nacalai Tesque Co. (Kyoto, Japan), Wako Pure Chemical Industries (Osaka, Japan), Sigma Chemical Co. (St. Louis, USA), and Sigma Chemical Co. (St. Louis, USA), respectively. Lutein was kindly provided from Koyo Mercantile Co. Ltd. (Tokyo, Japan). RAR antagonist, LE540, was kindly provided from Dr. Kagechika of Tokyo Medical and Dental University. All test chemicals were dissolved in dimethylsulfoxide (DMSO) (Nacalai Tesque, Kyoto, Japan) and stored at −80 °C until use. Antibody against ABCA1 was kindly provided from Dr. Yokoyama of Medical School of Nagoya City University.

2.2. Animals

Male C57BL/6J mice (6–8 weeks of age) were purchased from Nippon Charles River (Kanagawa, Japan). All animals were

kept in a temperature-controlled room (23 ± 1 °C) with a 12 h light/dark cycle, under specific-pathogen-free conditions and given a sterilized commercial diet (CE-2; Nippon Crea Co., Ltd., Shizuoka, Japan) and water *ad libitum* at the Laboratory Animal Center of Nagoya City University. All animal procedures were approved by the institutional animal care and use committee of Nagoya City University.

2.3. Yeast two-hybrid assay

Yeast expressing GAL4DBD-RARα or γ, GAL4DBD-RXRγ, GAL4AD-TIF2, and *lacZ* reporter plasmids were kindly provided by Dr. Nishikawa of Osaka University [32]. Yeast transformants were grown overnight at 30 °C with vigorous shaking in 5 ml of selective medium without leucine and tryptophan. The yeast was harvested by a centrifugation at 3000 × *g* for 15 min and suspended in fresh medium. The absorbance at 630 nm of the cell suspension was measured with U-2001 spectrophotometer (Hitachi High-Technologies Co., Tokyo, Japan). All assays were performed by using the yeast suspension with the absorbance at 630 nm of 0.6. Four hundred and fifty microliters aliquots of the yeast suspension were cultured in the presence of 5 μl of test compounds for 4 h at 30 °C with vigorous shaking. The 100 μl of chemically treated yeasts were incubated with 30 μl of Z buffer (0.1 M sodium phosphate (pH 7.9), 10 mM KCl, 1 mM $MgSO_4$) containing 4 mg/ml ZYMOLYASE®-20T (Seikagaku Co., Tokyo, Japan) for 30 min at 30 °C. The enzymatic reaction of β-galactosidase was initiated by the addition of 25 μl of 0.5 mg/ml chlorophenol Red β-D-galactopyranoside dissolved in 0.1 M sodium phosphate buffer (pH 7.9). When the red color developed, 50 μl of 2 M Na_2CO_3 were added to stop the reaction. The absorbances at 540 and 630 nm were measured and the activity was calculated according to the following equation: U = [(absorbance at 540 nm) − (absorbance at 630 nm)]/(absorbance at 630 nm at the start of the assay). β-Galactosidase activity is presented as the means ± S.D. of three determinations.

2.4. Binding assay using CoA-BAP system [33]

Binding assay was performed using Nu ligand kit (Microsystems, Kyoto, Japan) according to the manufacturer's instructions. Briefly, 100 μl of GST-fused nuclear receptor protein dissolved in 0.1 M sodium carbonate buffer (pH 6.8) was added to 96-well plate and incubated overnight at 4 °C. After washing the plate with 120 μl of wash buffer A, alkaline phosphatase-fused TIF2 protein dissolved in wash buffer A and test compounds dissolved in DMSO were added and incubated for 1 h at 4 °C. The plate was then washed with 120 μl of wash buffer B. The enzyme reaction was started at 30 °C by the addition of 100 μl of NPP solution. When the yellow color developed, the reaction was stopped by the addition of 25 μl of 0.5 M NaOH. The absorbance at 405 nm was measured with ARVO™ Wallac 1420 Multilabel Counter (Wallac, Finland).

2.5. Cell culture

Thioglycolate-elicited macrophages were prepared as previously described [34]. Briefly, C57BL/6J mice were intraperitoneally injected with 2 ml of 3% thioglycolate medium (Difco Laboratories, Detroit, MI, USA). Six days later, peritoneal macrophages were harvested from the abdominal cavity, seeded at a concentration of 2 × 10^6 cells/ml and maintained in RPMI1640 medium (Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin (Invitrogen), and 100 μg/ml of streptomycin (Invitrogen) for 2 h. After non-adherent cells were removed, the resulting macrophages were incubated with various concentrations of the test compounds in RPMI1640 medium supplemented with 0.1% bovine serum albumin for 8 and 20 h in order to prepare mRNA and protein fraction, respectively.

2.6. Western blot analysis

After 20 h incubation, macrophages were harvested and lysed by homogenization in 80 μl of lysis buffer containing 10 mM Tris-base, pH 8.0, 0.1% Triton X-100, 0.15 mM KCl, 5 mM mercaptoethanol, 1.3 mM EDTA, and protease inhibitor cocktail tablets (Roche Diagnostics) on ice. The lysates were palletized by centrifugation (12,000 × *g*, 15 min) at 4 °C, and the supernatant was assayed for protein concentration (Bradford method, Bio-Rad Laboratories). The lysates (protein 30 μg) were suspended in 0.9 M urea, 0.2% (v/v) Triton X-100, and 0.1% (w/v) dithiotheitol and supplied with 10% (w/v) lithium dodecylsulfate and then separated by 8% LDS-PAGE followed by transferring onto a polyvinylidene fluoride membrane (Millipore) in a transfer buffer (25 mM Tris, 192 mM glycine, 0.02% SDS, 20% methanol). The membrane was blocked for 3 h in a solution of 5% powdered skim milk in Tris-buffered saline (TBS) and then incubated with anti-ABCA1 antibody diluted 1:1000 in TBS containing 1% of skim milk and 0.05% Tween-20 overnight at 4 °C. The blot was washed in three changes of wash buffer (0.05% Tween-20 in TBS) and then incubated with alkaline phosphatase-conjugated anti-rabbit IgG antibody (Cell Signaling Technology, MA, USA) in 1% powdered skim milk and 0.05% Tween-20 in TBS for 1 h at room temperature. The blot was thoroughly washed in three changes of wash buffer, and ABCA1 was detected using CDP-Star (PE Biosystems, Bedford, MS, USA) as a substrate of alkaline phosphatase. Protein concentration was determined using LAS-3000mini (FUJIFILM, Tokyo, Japan).

2.7. Reverse transcription

After 8 h incubation, macrophages were harvested and total RNA from macrophages was extracted using a RNeasy™ mini kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's instruction. The resulted RNA was treated with DNase I (Invitrogen) to degrade contaminating DNA. The RNA was dissolved in diethyl pyrocarbonate-treated water and quantified by GeneQuant II (GE Healthcare Bio-Science Corp., NJ, USA). To prepare first strand cDNA, 500 ng of total RNA was reverse-transcribed using Revertra Ace (Toyobo Biochemicals, Tokyo, Japan) according to the manufacturer's instructions.

2.8. Quantitative RT-PCR

Reactions were prepared in 96-well optical grade PCR plate in a total of 50 μl containing the following components; 33 μg of cDNA dissolved in 25 μl of water, 25 μl of 2× TaqMan™ Universal Master Mix, 2.5 μl of TaqMan™ gene expression assays containing fluorogenic probes predesigned at Applied

Biosystems. Thermal cycling conditions consisted of an initial step of 2 min at 50 °C and then 10 min at 95 °C, followed by 40 cycles of 15 S at 95 °C and 1 min at 60 °C for ABCA1, ABCG1, and β-actin. Adjustments for baseline and threshold were performed according to the manufacturer's instructions. Levels of ABCA1 and ABCG1 mRNA expression were subsequently normalized relative to β-actin mRNA levels and calculated with delta–delta Ct method.

2.9. *Statistical analysis*

Data was represented as the mean ± S.D. or S.E. as described in the legends. Statistical significance was determined by Dunnett's test using Stat Light software. *P*-values less than 0.05 were considered significant.

3. Results

3.1. *RAR ligand activity using yeast two-hybrid assay*

The results of the binding activity against RARα and γ for the carotenoids and xanthopylls evaluated are shown in Fig. 1 for the yeast two-hybrid assay described in Section 2. atRA showed dose-dependent increase in β-galactosidase activity at the concentration of 10^{-10} to 10^{-8} M (Fig. 2A). Among carotenoids and xanthophylls tested, β-cryptoxanthin exhibited a dose-dependent increase in β-galactosidase activity in the concentration of 10^{-7} to 10^{-5} M. Lutein also showed increased β-galactosidase activity but at higher concentrations than β-cryptoxanthin. In contrast, β-carotene, zeaxanthin, astaxanthin, and lycopene did not exhibit significant activity. The transcription activation by β-cryptoxanthin and lutein was observed in both RARα and RARγ assays. When the agonist activity against RXRγ, which forms the heterodimer with RAR and transduces the signal, was determined, no activity was detected among any of the carotenoids and xanthophylls tested (Fig. 2B).

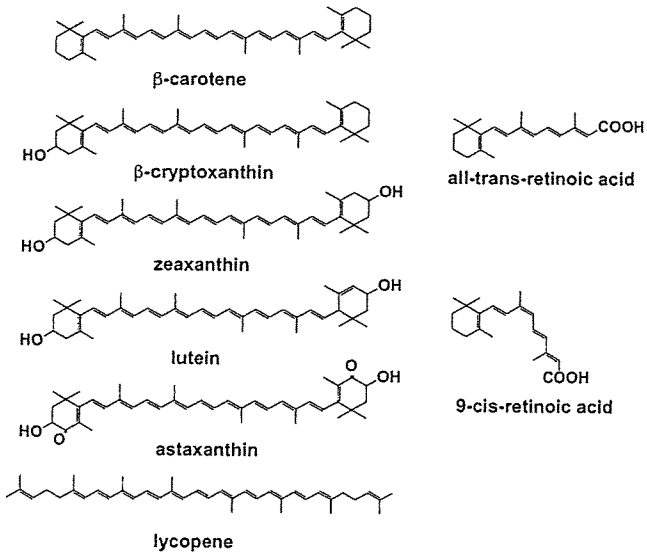

Fig. 1 – Structures of xanthophylls, carotenoids, and retinoic acid used in the present study.

3.2. *Effect of RAR pan-antagonist LE540 on RAR binding activity of β-cryptoxanthin and lutein*

To confirm whether β-galactosidase was induced by β-cryptoxanthin and lutein via RARα or RARγ, the effect of LE540, which is a pan-antagonist for RARs [35], was investigated. As shown in Fig. 3, β-cryptoxanthin induced β-galactosidase activity at the concentrations of 0.1 and 1 μM. This induction was completely inhibited by 10 μM LE540. Lutein induced β-galactosidase activity at 1 μM concentration and 10 μM LE540 abolished that induction. These results indicate that both β-cryptoxanthin and lutein induce β-galactosidase via RARα or γ in this yeast two-hybrid assay.

3.3. *Binding assay for RAR using CoA-BAP system*

To assess the agonist activity of β-cryptoxanthin and lutein and to preclude the possibility that β-cryptoxanthin metabolites or degradation products by yeast bind RARs, the binding activity to RAR was evaluated using the cell-free CoA-BAP system [33]. β-Cryptoxanthin and lutein were found to bind RARα in agreement with the result obtained in yeast two-hybrid assay. Conversely, zeaxanthin did not exhibit comparable binding activity in the CoA-BAP systems (Fig. 4) as was found in yeast two-hybrid assay. The β-cryptoxanthin dose where the binding activity was detected was three orders of magnitude higher than that of atRA. Additionally, when the binding activity to RXR was measured, no activity was found in β-cryptoxanthin and lutein. This corresponds to the results obtained from the yeast two-hybrid assay.

3.4. *Effect of β-cryptoxanthin on ATP-binding cassette transporters in macrophages*

Recently, ATP-binding cassette transporters A1 (ABCA1) and ABCG1 were reportedly induced in macrophages following the activation of RAR/RXR by retinoic acid [17]. To verify that β-cryptoxanthin acts as an RAR agonist in cells, the capacity of β-cryptoxanthin to induce ABCA1 and ABCG1 mRNAs in macrophages was evaluated. As shown in Fig. 5, β-cryptoxanthin increased mRNA levels of ABCA1 and ABCG1 dose-dependently, although the levels were less than those induced by atRA. Furthermore, the concentrations needed to induce both mRNAs were very similar to those found to induce effects in the yeast two-hybrid assay as well as in the CoA-BAP system. When ABCA1 protein levels were assessed by Western blot analysis, β-cryptoxanthin increased protein levels compared with vehicle-treated group, but less than atRA (Fig. 6A). In addition, the induction of ABCA1 protein by β-cryptoxanthin (5 μM) as well as atRA (5 μM) was completely abrogated by LE540 treatment (Fig. 6B). However, β-cryptoxanthin was not capable of increasing ABCG1 protein, although it increased ABCG1 mRNA levels (data not shown).

4. Discussion

In the present study, it has clearly demonstrated that β-cryptoxanthin and lutein, which are major nutrients in fruits and vegetables, are novel natural ligands for RARs and that β-

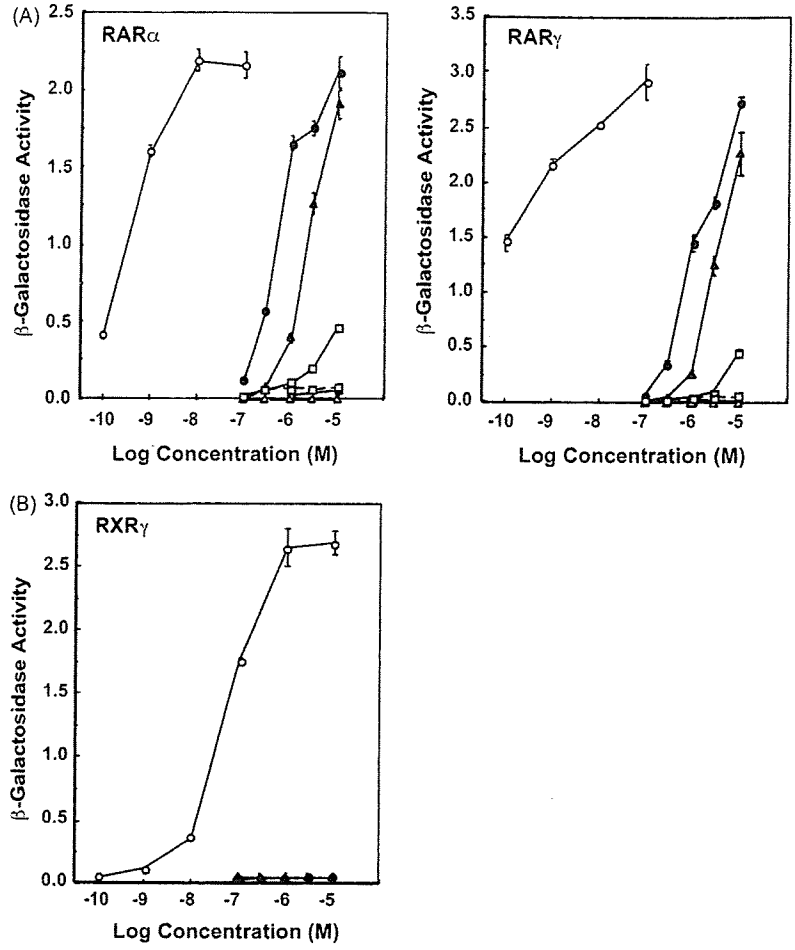

Fig. 2 – Yeast two-hybrid assay for RARα or RARγ activity. Yeast strain Y190 was transformed with GAL4 activation domain fused to TIF2 and GAL4 DNA binding domain fused to agonist-binding domain of RARα or RARγ. Chemicals were added to yeast cultures in doses ranging from 10^{-10} to 10^{-5} M. Following 4 h incubation the cultures were then assayed for β-galactosidase activity. Open circle in (A) and (B): atRA and 9-*cis* RA, respectively, closed circle: β-cryptoxanthin, open triangle: astaxanthin, closed triangle: lultein, open square: β-carotene, closed square: zeaxanthin, open square with broken line: lycopene. The values are represented as means ± S.E. of three determinants from a representative of three independent experiments, which showed similar results.

cryptoxanthin is a more potent ligand than lutein. Simultaneously, it has been shown that β-carotene, zeaxanthin, astaxanthin, and lycopene failed to show similar activity. An RAR pan-antagonist, LE540, completely abolished the β-crypotoxanthin- or lutein-dependent interaction between GAL4-RAR LBD and GAL4-TIF2 in the yeast two-hybrid assay, indicating that β-cryptoxanthin and lutein serve as an RAR agonist. The agonist activity was also supported by the finding that β-cryptoxanthin induces ABCA1 and ABCG1 mRNAs and ABCA1 protein in macrophages. To eliminate the possibility that metabolites or degradation products of β-cryptoxanthin act as RAR agonist, the binding assay was performed in cell-free CoA-BAP system. β-Cryptoxanthin exhibited binding activity against RAR LBD in a dose-dependent manner similar with the finding in the yeast two-hybrid assay. These data led to the conclusion that β-cryptoxanthin and lutein act as natural RAR agonists and may have preventive effects on atherosclerosis and restenosis.

β-Carotene is known to be cleaved in the intestinal mucosa at higher efficiency than any other organs. A responsible enzyme for cleaving β-carotene is β-carotene 15,15′-dioxygenase [36], which has a low level of activity toward provitamin A sources aside from β-carotene. The resulting all-*trans*-retinol (vitamin A) is first esterified to give an all-*trans*-retinyl ester, which is stored in the liver. In plasma, the vitamin A concentration is usually maintained at about 1 μM and the biologically active retinoid, atRA, is produced as the need arises in cells where the metabolic enzymes, aldehyde dehydrogenase and retinal dehydrogenase exist. The plasma concentration of atRA is therefore maintained at around a 12 nM level [37] and about 15% of this form is converted to 9-*cis* retinoic acid [38]. When considering the individual carotinoid and xanthopyll concentrations in plasma, β-cryptoxanthin has been shown to reach a concentration than other major carotenoids and xanthophylls, including β-carotene, α-carotene, lycopene, lutein, and zeaxanthin, assuming that the

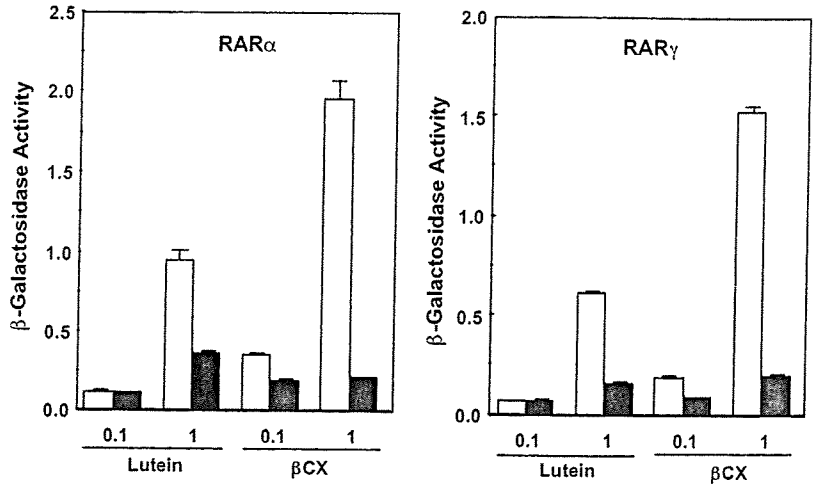

Fig. 3 – Effect of RAR pan-antagonist, LE540, on the transcriptional activity of β-cryptoxanthin or lutein against RARα or RARγ. Following 30 min incubation of 10 μM LE540 with yeast cultures, β-cryptoxanthin or lutein was added to yeast cultures at the indicated concentrations (μM) and incubated for 4 h. The cultures were then assayed for β-galactosidase activity. Open column: without LE540, closed column: with LE540. The values are represented as means ± S.E. of three determinants from a representative of three independent experiments, which showed similar results.

equal amounts of each of these carotenoid is present in the diet [39]. In actuality, β-cryptoxanthin reaches to 0.4–1.1 μM concentrations in the plasma after the intake of orange juice rich in cryptoxanthin [40,41]. In addition, the β-cryptoxanthin concentration in adipose tissue is also higher than that of other carotenoids and that the tissue distribution of β-cryptoxanthin is definitely different from that of atRA. The results of this study have shown that a 5×10^{-7} M of β-cryptoxanthin is sufficient to transactivate RAR and act as an RAR agonist, although the RAR-binding affinity of β-cryptoxanthin is three orders of magnitude lower than that of atRA. These results led to the hypothesis that the difference in the pharmacokinetics between β-cryptoxanthin and atRA makes β-cryptoxanthin an RAR agonist with physiological effects distinct from atRA. Although the preventive effects of vegetable and fruit intake against cardiovascular diseases have been considered based the known anti-oxidative effects of carotenoids found in vegetables and fruits, the result presented here strongly suggest that a β-cryptoxanthin signaling mechanism via RAR/RXR heterodimer contributes to the preventive effects by enhancing the expression of anti-atherogenic molecules such as ABCA1 and ABCG1.

This study did not reveal a structure–activity relationship that can explain why β-cryptoxanthin exhibited better binding activity to RAR in comparison to the other carotenoids and xanthopylls tested in this study. However, this difference may potentially be explained based upon the polarity and hydrophobicity properties of these compounds. It is reported that

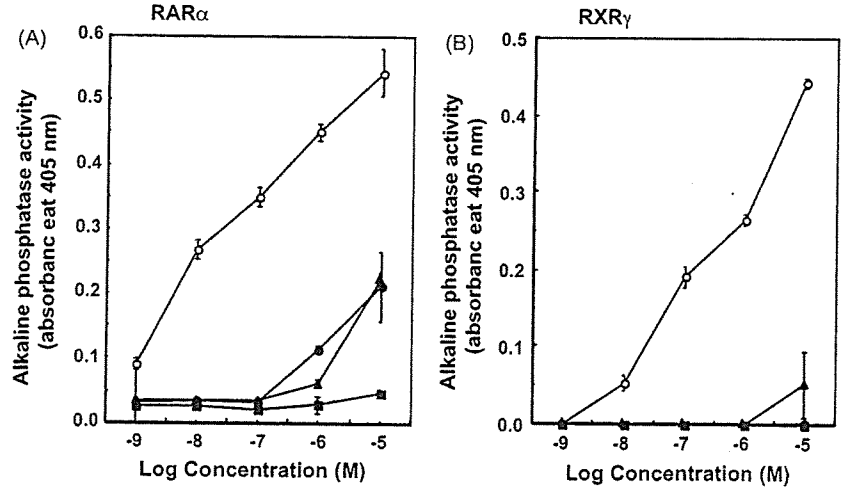

Fig. 4 – Binding activity of β-cryptoxanthin and lutein against RARγ in *in vitro* CoA-BAP system. Agonist-dependent interactions between GST-RARγ and TIF2-BAP were determined as alkaline phosphatase activity according to the method described in Section 2. Open circle in (A) and (B): atRA and 9-cis RA, respectively, closed circle: β-cryptoxanthin, closed triangle; lutein, closed square: zeaxanthin. The values are represented as means ± S.E. of three determinants.

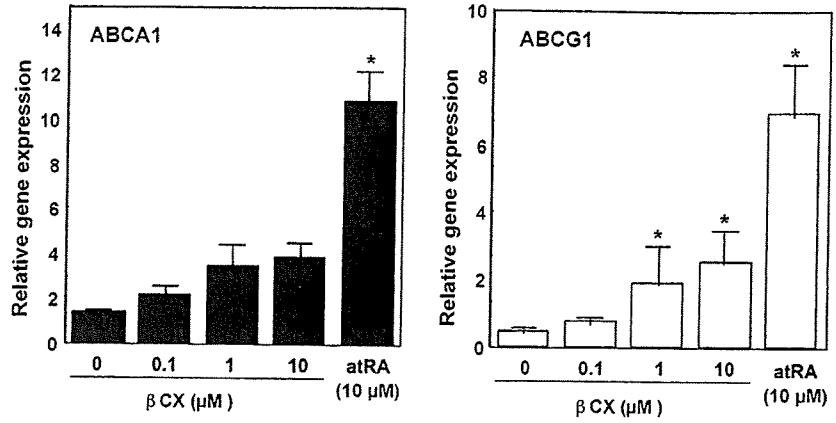

Fig. 5 – Effect of β-cryotoxanthin on induction of ABCA1 or ABCG1 mRNA in mouse peritoneal macrophages. Thioglycolate-elicited peritoneal macrophages were seeded at a concentration of 2.0×10^6 cells/ml and treated with β-cryptoxanthin or atRA at the indicated concentrations for 8 h. mRNA levels of ABCA1 and ABCG1 were determined by quantitative RT-PCR as described in Section 2. Data were normalized using the β-actin levels and are expressed relative to the cells treated with vehicle (DMSO). The values are represented as means ± S.D. of 3–4 culture dishes. Statistically significant differences from the cells treated with vehicle are indicated followed by Dunnett's test ($p < 0.05$).

lutein and zeaxanthin, which are dipolar xanthophylls, appear to adopt an orientation mainly perpendicular to membrane surfaces because the hydrophilic groups at the opposite ends of the molecule are attracted to the polar zones of the membrane at the membrane surfaces [42]. For carotenes lacking such polar groups, such as β-carotene and lycopene, orientation in the lipid membrane environment seems to be exclusively governed by van der Waals interactions with the hydrocarbon acyl chains of lipid molecules thereby resulting

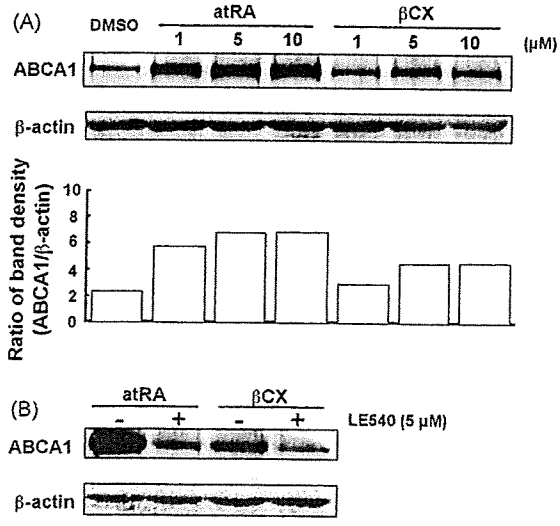

Fig. 6 – Induction of ABCA1 protein by β-cryptoxanthin. Thioglycolate-elicited peritoneal macrophages were seeded at a concentration of 2.0×10^6 cells/ml and treated with increasing concentrations of β-cryptoxanthin or atRA for 20 h in the absence (A) or presence (B) of an RAR pan-antagonist LE540 (5 μM). ABCA1 protein was determined by Western blot analysis as described in Section 2. The figure is a representative of three independent experiments, which show similar results.

in random orientations of these carotenoid in membranes. In the case of β-cryptoxanthin, a monopolar xanthophyll, the hydrophilic portion seems to be attracted to the membrane surface with hydrophobic end floating in the core of the membrane [43]. In fact, although RAR does not exist in membrane, such difference in molecular polarity may influence the interaction between ligands and RAR in RAR ligand-binding pocket. Recently, a zeaxanthin-binding protein was isolated and identified as a Pi isoform of glutathione S-transferase [44]. Thus, there is a possibility that a β-cryptoxanthin-specific protein exists and transactivates RAR in a ligand-dependent manner. Of particular interest from the present work is that β-carotene failed to show any ligand activity at physiological or pharmacological concentrations. In addition, it has been reported that the carboxyl group of retinoid cannot be replaced by typical bioisosteric functional groups, such as sulfonamide and tetrazole [45]. This suggests that β-cryptoxanthin may possess a structural uniqueness as a RAR ligand.

To date, there are only a limited numbers of reports concerning the biological activities of β-cryptoxanthin. Among them, β-cryptoxanthin has been found to possess a unique anabolic effect on bone calcification [46], stimulation of bone formation, and inhibition of bone resorption [47]. The authors of those resports denied the possibility that β-cryptoxanthin shows such effects via RAR activation, although the evidence is not completely clear. However, Lian et al. reported that β-cryptoxanthin suppresses the growth of human bronchial cells by upregulating RARβ expression [48]. They also indicated that β-cryptoxanthin can transactivate RAR-mediated transcription activity of the retinoic acid response element. However, they concluded that the metabolites of β-cryptoxanthin might induce RARβ expression based upon the need for a higher effective dose of β-cryptoxanthin than atRA for the induction of RARβ expression or transactivation of RARs. In contrast, the use of the CoA-BAP system employed in the present study to assay the ligand

activity against RARα reduces the potential that metabolites or degradation products of β-cryptoxanthin are responsible for such activity. Furthermore, in yeast two-hybrid assay, β-cryptoxanthin induced β-galactosidase via RARs at the concentration of 5×10^{-7} M. Furthermore, the RAR pan-antagonist, LE540, effectively inhibited β-galactosidase induction by β-cryptoxanthin. Taken together, these results indicate that β-cryptoxanthin itself, not its metabolites, acts as an RAR ligand and stimulates RAR-mediated transcription activity at physiological concentrations.

Considering that the prevalence of acute and chronic side effects has limited wide applicability of vitamin A and its retinoic acid derivatives as therapeutic drugs of choice, novel natural ligands against RAR should pave the way for not only understanding the mechanism underlying the pleiotropic effects of RA, but also developing the therapeutic agents with fewer side effects for atherosclerosis, cancer and other related conditions. In this respect, β-cryptoxanthin appears to be a molecule that warrants further investigation.

Recent epidemiological studies reveal that a moderate increase in β-cryptoxanthin intake is associated with a reduced risk of developing rheumatoid arthritis and lung cancer [49,50]. The results reported here led to the hypothesis that β-cryptoxanthin is capable of stimulating differentiation of lung cancer cells and modulating immune response through Th2 cells via RAR. Epidemiologic studies indicate that appropriate vegetable and fruit intake may be helpful in preventing cancer and cardiovascular diseases. Given the fact that vegetables and fruits contain β-cryptoxanthin, this molecule may indeed be the source of that anti-atherogenic and anti-tumor activity by acting through an RAR signaling mechanism.

In conclusion, the present study has shown that β-cryptoxanthin, a xanthophyll, exhibits agonist activity against RAR as well as exhibiting anti-atherogenic effect on macrophages by inducing ABCA1 and G1 expressions. Since natural RAR ligands other than retinol metabolites have not previously been found, β-cryptoxanthin, a provitamin A carotenoid that exhibits RAR agonist activity and acts as an RAR agonist which has hydrophobicity and exhibits a metabolic fate different from atRA, should be potential candidate for preventive or therapeutic agents against cardiovascular diseases.

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REFERENCES

[1] Voutilainen S, Nurmi T, Mursu J, Rissanen TH. Carotenoids and cardiovascular health. Am J Clin Nutr 2006;83:1265–71.

[2] Joshipura KJ, Ascherio A, Manson JE, Stampfer MJ, Rimm EB, Speizer FE, et al. Fruit and vegetable intake in relation to risk of ischemic stroke. JAMA 1999;282:1233–9.

[3] Joshipura KJ, Hu FB, Manson JE, Stampfer MJ, Rimm EB, Speizer FE, et al. The effect of fruit and vegetable intake on risk for coronary heart disease. Ann Intern Med 2001;134:1106–14.

[4] Bazzano LA, He J, Ogden LG, Loria CM, Vupputuri S, Myers L, et al. Fruit and vegetable intake and risk of cardiovascular disease in US adults: the first national health and nutrition examination survey epidemiologic follow-up study. Am J Clin Nutr 2002;76:93–9.

[5] Hennekens CH, Buring JE, Manson JE, Stampeer M, Rosner B, Cook NR, et al. Lack of effect of long-term supplementation with beta carotene on the indidence of malignant neoplasms and cardiovascular disease. N Engl J Med 1999;334:1145–9.

[6] Greenberg ER, Baron JA, Karagas MR, Stukel TA, Nierenberg DW, Stevens MM, et al. Mortality associated with low plasma concentration of beta carotene and the effect of oral supplementation. JAMA 1996;275:699–703.

[7] Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. N Engl J Med 2005;352:1685–95.

[8] Ross R. Atherosclerosis—an inflammatory disease. N Engl J Med 1999;340:115–26.

[9] Hansson GK, Libby P, Schönbeck U, Yan ZQ. Innate and adaptive immunity in the pathogenesis of atherosclerosis. Circ Res 2002;91:281–91.

[10] DeRose JJJr, Madigan J, Umane JP, Prystowsky JH, Nowygrod R, Oz MC, et al. Retinoic acid suppresses intimal hyperplasia and prevents vessel remodeling following arterial injury. Cardiovasc Surg 1999;7:633–9.

[11] Miano JM, Kelly LA, Artacho CA, Nuckolls TA, Piantedosi R, Blaner WS. All-*trans*-retinoic acid reduces neointimal formation and promotes favorable geometric remodeling of the rat carotid artery balloon withdrawal injury. Circulation 1999;98:1219–27.

[12] Lee CW, Park SJ, Park SW, Kim JJ, Hong MK, Song JK. All-*trans*-retinoic acid attenuates neointima formation with acceleration of reendothelialization in balloon-injured rat aorta. J Korean Med Sci 2000;15:31–6.

[13] Herdeg C, Oberhoff M, Baumbach A, Schroeder S, Leitritz M, Blattner A, et al. Effects of local all-trans-retinoic acid delivery on experimental atherosclerosis in the rabbit carotid artery. Cardiovasc Res 2003;57:544–53.

[14] Neuville P, Yan Zq, Gidlöf A, Pepper MS, Hansson GK, Gabbiani ZG, et al. Retinoic acid regulates arterial smooth muscle cell proliferation and phenotypic features *in vivo* and *in vitro* through an RAR-a-dependent signaling pathway. Arterioscler Throm Vasc Biol 1999;19:1430–6.

[15] Miano JM, Topouzis S, Majesky MJ, Olson EN. Retinoid receptor expression and all-trans retinoic acid-mediated growth inhibition in vascular smooth muscle cells. Circulation 1996;93:1886–95.

[16] Kosaka C, Sasaguri T, Komiyama Y, Takahashi H. All-trans retinoic acid inhibits vascular smooth muscle cell proliferation targeting multiple genes for cyclins and cyclin-dependent kinases. Hypertens Res 2001;24:579–88.

[17] Costet P, Lalanne F, Gerbod-Giannone MC, Molina JR, Fu X, Lund EG, et al. Retinoic acid receptor-mediated induction of ABCA1 in macrophages. Mol Cell Biol 2003;23:7756–66.

[18] Takeda N, Manabe I, Shindo T, Iwata H, Iimuro S, Kagechika H, et al. Synthetic retinoid Am80 reduces scavenger receptor expression and atherosclerosis in mice by inhibiting IL-6. Arterioscler Thromb Vasc Biol 2006;26:1177–83.

[19] Langmann T, Liebisch G, Moehle C, Schifferer R, Dayoub R, Heiduczek S, et al. Gene expression profiling identifies retinoids as potent inducers of macrophage lipid efflux. Biochim Biophys Acta 2005;1740:155–61.

[20] Iwata M, Eshima Y, Kagechika H. Retinoic acids exert direct effects on T cells to suppress T_h1 development and enhance T_h2 development via retinoic acid receptors. Int Immunol 2003;15:1017–25.
[21] Hoag KA, Nashold FE, Goverman J, Hayes C. Retinoic acid enhances the T helper 2 cell development that is essential for robust antibody responses through its action on antigen-presenting cells. J Nutr 2002;132:3736–9.
[22] David M, Hodak E, Lowe NJ. Adverse effects of retinoids. Med Toxicol Adverse Drug Exp 1988;3:273–88.
[23] Frankel SR, Eardley A, Lauwers G, Weiss M, Warrell Jr RP. The "retinoic acid syndrome" in acute promyelocytic leukemia. Ann Intern Med 1992;117:292–6.
[24] Petkovich M, Brand NJ, Krust A, Chambon P. A human retinoic acid receptor which belongs to the family of nuclear receptors. Nature 1987;330:444–50.
[25] Giguere V, Ong ES, Segui P, Evans RM. Identification of a receptor for the morphogen retinoic acid. Nature 1987;330:624–9.
[26] Chambon P. A decade of molecular biology of retinoic acid receptors. FASEB J 1996;10:940–54.
[27] Uppenberg J, Svensson C, Jaki M, Bertilsson G, Jendeberg L, Berkenstam A. Crystal structure of the ligand binding domain of the human nuclear receptor PPARγ. J Biol Chem 1998;273:31108–12.
[28] Kim KR, Lee JH, Kim SJ, Rhee SD, Jung WH, Yang SD, et al. KR-62980: a novel peroxisome proliferator-activated receptor γ agonist with weak adipogenic effects. Biochem Pharmacol 2006;72:446–54.
[29] Allen T, Zhang F, Moodie SA, Clemens LE, Smith A, Gregoire F, et al. Halofenate is a selective peroxisome proliferators-activated receptor γ modulator with antidiabetic activity. Diabetes 2006;55:2523–33.
[30] Napoli JL. Biosynthesis and metabolism of retinoid acid: roles of CRBP and CRABP in retinoic acid homeostasis. J Nutr 1993;123:362–6.
[31] Heyman RA, Mangelsdorf DJ, Dyck JA, Stein RB, Eichele G, Evans RM, et al. 9-cis retinoic acid is a high affinity ligand for the retinoid X receptor. Cell 1992;68:397–406.
[32] Nishikawa J, Saito K, Goto J, Dakeyama F, Matsuo M, Nishihara T. New screening methods for chemicals with hormonal activities using interaction of nuclear hormone receptor with coactivator. Toxicol Appl Pharmacol 1999;154:76–83.
[33] Kanayama T, Mamiya S, Nishihara T, Nishikawa J. Basis of a high-throughput method for nuclear receptor agonists. J Biochem 2003;133:791–7.
[34] Ono Y, Inoue M, Mizukami H, Ogihara Y. Suppressive effect of Kanzo-bushi-to, a Kampo medicine, on collagen-induced arthritis. Biol Pharm Bull 2004;27:1406–13.
[35] Umemiya H, Fukasawa H, Ebisawa M, Eyrolles L, Kawachi E, Eisenmann G, et al. Regulation of retinoidal actions byu doazepinylbenzoic acids. Retinoid synergists which activate the RXR-RAR heterdimers. J Med Chem 1997;40:4222–34.
[36] Redmond TM, Gentleman S, Duncan T, Yu S, Wiggert B, Gantt E, et al. Identification, expression, and substrate specificity of a mammalian β-carotene 15, 15'-dioxygenase. J Biol Chem 2001;276:6560–5.
[37] De Leenheer AP, Lambert WE, Claeys I. All-trans-retinoic acid: measurement of reference values in human serum by high performance liquid chromatography. J Lipid Res 1982;23:1362–7.
[38] Urbach J, Rando RR. Isomerization of all-trans retinoic acid to 9-cis-retinoic acid. Biochem J 1994;299:459–65.
[39] El-Sohemy A, Baylin A, Kabagambe E, Ascherio A, Spiegelman D, Campos H. Individual carotenoid concentrations in adipose tissue and plasma as biomarkers of dietary intake. Am J Clin Nutr 2002;76:172–9.
[40] Wingerath T, Stahl W, Sies H. β-Cryptoxanthin selectively increases in human chylomicrons upon ingestion of tangerine concentrate rich in β-cryptoxanthin esters. Arch Biochem Biophys 1995;324:385–90.
[41] Riso P, Visioli F, Gardana C, Grande S, Brusamolino A, Galvano F, et al. Effects of blood orange juice intake on antioxidant bioavailability and on different markers related to oxidative stress. J Agric Food Chem 2005;53:941–7.
[42] Gruszecki WI, Strzalka K. Carotenoids as modulators of lipid membrane physical properties. Biochim Biophys Acta 2005;1740:108–15.
[43] Wisniewska A, Widomska J, Subczynski WK. Carotenoid-membrane interactions in liposomes: effect of dipolar, monopolar, and nonpolar carotenoids. Acta Biochim Pol 2006;53:475–84.
[44] Bhosale P, Larson AJ, Frederick JM, Southwick K, Thulin CD, Bernstein PS. Identification and characterization of a Pi isoform of glutathione S-transferase (GSTP1) as a zeaxanthin-binding protein in the macula of the human eye. J Biol Chem 2004;279:49447–54.
[45] Kagechika H, Shudo K. Synthetic retinoids: recent developments concerning structure and clinical utility. J Med Chem 2005;48:5875–83.
[46] Yamaguchi M, Uchiyama S. Effect of carotenoid on calcium content and alkaline phosphatase activity in rat femoral tissues in vitro: the unique anabolic effect of β-cryptoxanthin. Biol Pharm Bull 2003;26:1188–91.
[47] Uchiyama S, Yamaguchi M. Inhibitory effect of β-cryptoxanthin on osteoclast-like cell formation in mouse marrow cultures. Biochem Pharmacol 2004;67:1297–305.
[48] Lian F, Hu KQ, Russell RM, Wang XD. β-Cryptoxanthin suppresses the growth of immortalized human bronchial epithelial cells and non-small-cell lung cancer cells and up-regulates retinoic acid receptor β expression. Int J Cancer 2006;119:2084–9.
[49] Yuan JM, Stram DO, Arakawa K, Lee HP, Yu MC. Dietary cryptoxanthin and reduced risk of lung cancer: the siggapore Chinese health study. Cancer Epidemiol Biomark Prev 2003;12:890–8.
[50] Pattison DJ, Symmons DPM, Welch MLA, Bingham SA, Day NE, Silman AJ, et al. β-cryptoxanthin and inflammatory polyarthritis: results from a population-based prospective study. Am J Clin Nutr 2005;82:451–5.

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Histone acetyltransferase MOZ acts as a co-activator of Nrf2–MafK and induces tumour marker gene expression during hepatocarcinogenesis

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HATs (histone acetyltransferases) contribute to the regulation of gene expression, and loss or dysregulation of these activities may link to tumorigenesis. Here, we demonstrate that expression levels of HATs, p300 and CBP [CREB (cAMP-response-element-binding protein)-binding protein] were decreased during chemical hepatocarcinogenesis, whereas expression of MOZ (monocytic leukaemia zinc-finger protein; MYST3) – a member of the MYST [MOZ, Ybf2/Sas3, Sas2 and TIP60 (Tat-interacting protein, 60 kDa)] acetyltransferase family – was induced. Although the MOZ gene frequently is rearranged in leukaemia, we were unable to detect MOZ rearrangement in livers with hyperplastic nodules. We examined the effect of MOZ on hepatocarcinogenic-specific gene expression. GSTP (glutathione S-transferase placental form) is a Phase II detoxification enzyme and a well-known tumour marker that is specifically elevated during hepatocarcinogenesis. GSTP gene activation is regulated mainly by the GPE1 (GSTP enhancer 1) enhancer element, which is recognized by the Nrf2 (nuclear factor-erythroid 2 p45 subunit-related factor 2)–MafK heterodimer. We found that MOZ enhances GSTP promoter activity through GPE1 and acts as a co-activator of the Nrf2–MafK heterodimer. Further, exogenous MOZ induced GSTP expression in rat hepatoma H4IIE cells. These results suggest that during early hepatocarcinogenesis, aberrantly expressed MOZ may induce GSTP expression through the Nrf2-mediated pathway.

Key words: glutathione S-transferase placental form (GSTP), hepatocarcinogenesis, histone acetyltransferase (HAT), MafK, monocytic leukaemia zinc-finger protein (MOZ), nuclear factor-erythroid 2 p45 subunit-related factor 2 (Nrf2).

INTRODUCTION

Acetylation is an important post-translational modification known to occur in histones, transcription factors and other proteins [1]. Histone acetylation is catalysed by HATs (histone acetyltransferases), which transfer an acetyl group from acetyl-CoA to lysine residues in histones. Lysine acetylation destabilizes the nucleosome structure and promotes the accessibility of transcription factors to a genetic locus. In agreement with these phenomena, acetylated chromatin has been associated with a transcriptionally activated state [2].

The well-characterized transcriptional co-activators CBP [CREB (cAMP-response-element-binding protein)-binding protein], p300, GCN5 (positive general control of transcription-5), and P/CAF (p300/CBP-associated factor) have intrinsic HAT activity [3–6]. HATs are divided into several groups on the basis of their similarity in homologous regions including acetyl-CoA-binding motifs [1,7]. For example, p300 and CBP, and P/CAF and GCN5 share a remarkable degree of similarity throughout their sequences respectively, and they play distinct but functionally overlapping roles [1,8,9]. Another group of evolutionarily related HATs is the MYST [MOZ (monocytic leukaemia zinc-finger protein), Ybf2/Sas3, Sas2 and TIP60 (Tat-interacting protein, 60 kDa)] family. MYST proteins not only contribute to transcriptional activation, but they also have diverse roles in various nuclear processes, including cell-cycle progression, DNA repair, DNA replication and gene silencing [10–16].

Recent studies indicate that some HATs play roles in tumour suppression and that loss or misregulation of these activities may lead to cancer [17]. Development of liver cancer is controlled by several transcriptional factors, such as c-Jun, Foxm1b (forkhead box m1b) and p53 [18,19]. These factors are acetylated by p300 and CBP, which thereby modulate the transcriptional activity of these factors [1,19]. Hyperacetylation of histones in the promoter region of c-Jun is detected [20]. In addition, p53 recruits p300 to nucleosomal histones within the p21 promoter *in vitro* [21]. These results suggest that HATs may be involved in hepatocarcinogenesis, but the underlying mechanism has not been addressed. Here, we show that MOZ (also known as MYST3), a member of the MYST family of HATs, is induced during hepatocarcinogenesis. MOZ frequently is rearranged in leukaemia [10,22–27], and it regulates transcription mediated by the haemopoietic transcriptional factor AML1 (acute myeloid leukaemia 1) and the MOZ fusion protein, which is generated by translocation, down-regulates in haemopoiesis and leads to leukaemogenesis [28].

GSTP [GST (glutathione S-transferase) placental form] is a Phase II detoxification enzyme and a well-known tumour marker that is specifically elevated during chemical hepatocarcinogenesis in the rat [29]. GSTP gene expression is regulated mainly through

Abbreviations used: AAF, 2-acetylaminofluorene; AML1, acute myeloid leukaemia 1; bZIP, basic region leucine zipper; CREB, cAMP-response-element-binding protein; CBP, CREB-binding protein; DEN, diethylnitrosoamine; DTT, dithiothreitol; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCN5, positive general control of transcription-5; GST, glutathione S-transferase; GSTP, GST placental form; GPE, GSTP enhancer; HA, haemagglutinin; HAT, histone acetyltransferase; HBO1, HAT binding to ORC1 (origin recognition complex subunit 1); ING, inhibitor of growth; MOZ, monocytic leukaemia zinc-finger protein; MORF, MOZ related factor; MYST, MOZ, Ybf2/Sas3, Sas2 and TIP60; Nrf2, nuclear factor-erythroid 2 p45 subunit-related factor 2; ORF, open reading frame; P/CAF, p300/CBP-associated factor; PH, partial hepatectomy; PHD, plant homeodomain; RT, reverse transcriptase; TIF2, transcriptional intermediary factor 2, TIP60, Tat-interacting protein, 60 kDa; TRE, PMA ('TPA')-responsive element.

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GPE (GSTP enhancer), located approx. 2.5 kb upstream from the cap site, and the silencer [30,31]. GPE1, a strong enhancer element in GPE, is responsible for GSTP gene expression during hepatocarcinogenesis *in vivo* [32,33]. Recently, we showed that a heterodimer comprising Nrf2 (nuclear factor-erythroid 2 p45 subunit-related factor 2) and MafK binds to GPE1 and enhances GSTP promoter activity [34]. Nrf2, a member of bZIP (basic region leucine zipper) family of transcription factors, induces Phase II detoxifying and antioxidative genes [35]. Nrf2 plays a crucial role in early defence against chemical stress and carcinogenesis [36].

To characterize the roles of HATs during hepatocarcinogenesis, we examined their expression profiles and showed that expression of MOZ was induced under these conditions. Further, we found that MOZ acted as a co-activator of the Nrf2–MafK heterodimer and induced expression of GSTP. These results suggest that MOZ induces GSTP expression through the Nrf2-mediated pathway during early hepatocarcinogenesis.

EXPERIMENTAL

Chemical hepatocarcinogenesis of rats

Carcinogenic experiments were performed according to the Solt–Farber protocol [37]. Experiments were initiated by intraperitoneal injection of DEN (diethylnitrosoamine; 200 mg/kg) into 5-week-old Wister rats. After the animals had been fed basal diets for 2 weeks, diets were changed to basal diets containing 0.02 % AAF (2-acetylaminofluorene). Three weeks after the DEN injection, a PH (partial hepatectomy) was performed; livers were extirpated 7 weeks after the DEN injection. Control rats were injected with saline and fed basal diets. All animal care and handling procedures were approved by the Animal Care and Use Committee of Osaka University.

Preparation of nuclear extracts, cytosol fractions and RNA from rat liver

Procedures for preparation of nuclear extracts and cytosol fractions from rat liver were described previously [38]. Livers were homogenized in a sucrose-containing buffer, and nuclei were purified by centrifugation. Nuclear proteins were extracted with 0.55 M KCl and centrifuged at 40000 *g* for 60 min at 4 °C. The supernatants were used for the HAT assay and Western blot analysis. Total RNA was isolated using TRIzol® reagent (Invitrogen, Carlsbad, CA, U.S.A.) in accordance with the manufacturer's recommendations.

Western blotting and antibodies

Proteins were resolved using SDS/PAGE, transferred to nitrocellulose or PVDF membrane and detected using the ECL® (enhanced chemiluminescence) Western blotting analysis detection system (Amersham Biosciences, Piscataway, NJ, U.S.A.). For the generation of antibodies against the N- and C-terminal regions of MOZ, nucleotides corresponding to amino acid residues 1–331 and 1717–1998 respectively were cloned into pET-28a (Novagen, Darmstadt, Germany). The resulting His_6-tagged fusion polypeptides were expressed in bacteria and purified over nickel-nitrilotriacetic acid–agarose (Qiagen, Hilden, Germany). These proteins were injected into rabbits, and antibodies were affinity-purified using Protein A–Sepharose (Amersham Biosciences). The anti-P/CAF antibody was a gift from Dr Y. Nakatani (Harvard Medical School, Boston, MA, U.S.A.). The following antibodies were commercially available: anti-p300 (N-15, Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), anti-CBP (A-22, Santa Cruz Biotechnology), anti-GCN5 (N-18, Santa Cruz Biotechnology), anti-TIP60 (Upstate Biotechnology, Lake Placid, NY, U.S.A.), anti-MORF (MOZ-related factor; C-15, Santa Cruz Biotechnology), anti-MYST (Upstate Biotechnology), anti-GSTP (Biotrin, Dublin, Ireland), anti-HA (haemagglutinin) (6B12, Babco, Berkeley, CA, U.S.A.), and anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (MAB374, Chemicon, Temecula, CA, U.S.A.).

Plasmid construction

The rat MOZ expression plasmid pCI-MOZ has been described previously [39]. Mutants within the PHD (plant homeodomain) finger and the MYST regions of the *Moz* gene (pCI-MOZ-PHDmut and pCI-MOZ-MYSTmut) were generated using the QuikChange® site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) following the manufacturer's recommended protocols. All mutations were verified by sequencing over the region of change. For construction of Myc-tagged MOZ-expressing plasmids, the MOZ ORF (open reading frame) was subcloned into the EcoRI–NotI site of pCMV-Myc (Clontech, Franklin Lakes, NJ, U.S.A.). For construction of −2.5GST-luciferase, the fragment from −2.5 kb to −91 kb of the GSTP gene [30] was inserted into the SacI site of −91GST-luciferase [38]. To generate −2.15GST-luciferase (the GPE deletion reporter plasmid), −2.5GST-luciferase was digested with SmaI and AccI, blunted with Klenow fragment (Toyobo, Osaka, Japan), and then self-ligated. The Nrf2 expression plasmid (pAβ2-Nrf2), including the human β-actin promoter and enhancer, and GPE1 reporter plasmid (GPE1-luciferase) were described previously in [40]. The HA-tagged rat MafK expression plasmid (pCMV-HA-MafK) was generated by PCR amplification of the MafK ORF [40] using primers that incorporate SalI and NotI at the 5′ and 3′ ends respectively. The PCR product was cloned into the SalI–NotI site of pCMV-HA (Clontech). The non-tagged MafK expression plasmid pRSV-MafK contained MafK cDNA controlled by the *Rous sarcoma* virus long terminal repeat. For construction of MafK/GEX-KG, MafK cDNA was cloned into pGEX-2T (Amersham Biosciences).

Cell culture

Rat hepatoma H4IIE cells and mouse embryonic carcinoma F9 cells were maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) FBS (fetal bovine serum). HeLa cells were cultured in minimal essential medium supplemented with 10 % FBS.

Transfection and reporter gene assays

Transfection of H4IIE and F9 cells was performed using FuGENE™ 6 (Roche, Indianapolis, IN, U.S.A.) in accordance with the manufacturer's instructions. For H4IIE cells, all transfections included 100 ng of the reporter plasmid, with or without 1 μg of the MOZ expression plasmid (pCI-MOZ). The amount of plasmid in the transfection was kept constant by using empty pCI vector. Transfectants were harvested 48 h after transfection. The luciferase assay was performed as described previously [38] and protein concentrations were determined by the method of Bradford. Luciferase activities were normalized to the protein amount. In some experiments, the transfection efficiency was checked by co-transfection with pRSV-GAL, a eukaryotic expression plasmid that contained the *Escherichia coli* β-galactosidase structural gene controlled by the *Rous sarcoma* virus long terminal repeat. β-Galactosidase activity was assayed as described in [38]. We confirmed that the variation of transfection efficiency was <15 %. Relative luciferase activity was estimated by the luciferase activity from −2.5GST-luciferase in the absence of MOZ.

For F9 cells, all transfections contained 100 ng of reporter plasmid (GPE1-luciferase) and 5 ng of *Renilla* luciferase plasmid phRL-tk (Promega, Madison, WI, U.S.A.) as an internal control to normalize for transfection efficiency, with or without 1 μg of the MOZ expression plasmid (pCI-MOZ) in the presence or absence of 5 ng of the Nrf2 expression plasmid (pAβ2-Nrf2). The amount of plasmid in the transfection was kept constant by using empty pCI vector. At 48 h after transfection, cells were harvested and assayed for luciferase activity using the Dual-luciferase Reporter Assay System (Promega) in accordance with the manufacturer's recommendations. Reported values are relative to the activity of GPE1-luciferase without transfection of Nrf2 and MOZ. All experiments were repeated at least three times with two or three different preparations of DNA.

GST pull-down assay

The recombinant plasmid was transformed into BL21(DE3)pLysS cells. Transformants were grown overnight at 30 °C in Luria–Bertani medium containing 100 μg/ml ampicillin. The culture then was diluted 25-fold and grown to an attenuance (D_{600}) of 0.4; at that time, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM. The cells were allowed to grow for an additional 4.5 h and then were harvested by centrifugation; resuspended in a buffer containing 0.15 M KCl, 50 mM Tris (pH 8.0), 10 % (v/v) glycerol, 0.1 % Tween 20, 1 mM DTT (dithiothreitol) and 1 mM PMSF, and disrupted by sonication. After centrifugation at 7000 *g* for 10 min, the supernatant was cross-linked to glutathione–Sepharose 4B with dimethylpimelimidate. ^{35}S-labelled MOZ proteins were produced using pCI-MOZ, pCI-MOZ-PHDmut and pCI-MOZ-MYSTmut as templates by *in vitro* transcription–translation with the TNT T7-coupled reticulocyte lysate system (Promega). A 5 μl aliquot of the reticulocyte lysate reaction containing ^{35}S-labelled MOZ proteins was incubated for 3 h at 4 °C in a buffer containing 0.15 M KCl, 50 mM Tris (pH 7.6), 10 % (v/v) glycerol, 1 mM DTT and 1 mM PMSF with GST fusion proteins. After extensive washes, bound proteins were separated by SDS/PAGE and detected by autoradiography.

Immunoprecipitation assay

Myc-tagged MOZ expression plasmid (pCMV-Myc-MOZ) was co-transfected into HeLa cells with HA-tagged MafK (pCMV-HA-MafK) or non-tagged MafK (pRSV-MafK) by the calcium phosphate co-precipitation method [41]. The cells were harvested 48 h after transfection, and nuclear extracts from the transfected HeLa cells were prepared as described in [38]. Nuclear extracts were diluted by adding nuclear lysis buffer containing 20 mM Hepes (pH 7.9), 1 mM EDTA, 0.5 mM spermidine, 1 mM DTT, 10 % glycerol, 1 mM PMSF, 1 μg/ml pepstatin A and 1 μg/ml leupeptin (final KCl concentration, 0.15 M). To immunoprecipitate HA-tagged protein, we incubated extracts with anti-HA antibody immobilized on Sepharose beads overnight at 4 °C. For control experiments, control mouse IgG coupled with Sepharose was used. After extensive washes, bound proteins were separated by SDS/PAGE and detected by Western blotting.

Induction of endogenous GSTP expression by MOZ in rat hepatoma H4IIE cells

Rat hepatoma H4IIE cells were transfected with various amounts of the MOZ expression plasmid pCI-MOZ by using the FuGENE™ 6 reagent in 35 mm plates. The total amount of plasmid DNA was adjusted by supplementing with empty pCI vector to 1 μg. After 36 h, cell lysates were prepared with a buffer comprising 25 mM Tris phosphate (pH 7.8), 2 mM cyclohexane-1,2-diaminetetra-acetic acid, 10 % glycerol, 2 mM DTT and 1 % Triton X-100. Cell lysates were separated by SDS/PAGE (15 % gel), and expression of endogenous GSTP and GAPDH was detected by Western blotting.

RESULTS

Activity and expression profiles of HATs during hepatocarcinogenesis

To evaluate the activity and expression profiles of HATs during hepatocarcinogenesis, we performed chemical carcinogenesis in the rat liver in accordance with the Solt–Farber protocol [37]. This model of liver chemical carcinogenesis is a widely used system for the study of molecular and cellular processes leading to cancer. In this protocol, rats were fed a combination of DEN and AAF and then underwent PH. At the end of 7 weeks, the livers had large numerous hyperplastic nodules, and the rats were killed (Figure 1A). We prepared four types of control experiments: rats underwent saline injection; were injected with DEN; underwent AAF feeding; underwent PH but were not treated with DEN and AAF. We checked the reproducibility of the carcinogenic experiments. Western blotting analysis of the cytosol fractions with an anti-GSTP antibody revealed that GSTP was induced at 7 weeks after combined treatment with DEN, AAF and PH, but no GSTP was detected in any of the control rats (Figure 1B).

We first investigated the HAT activity of nuclear extracts during hepatocarcinogenesis. The assay using core histones or nucleosome histones as substrates revealed that HAT activity in livers with hyperplastic nodules was indistinguishable from that in control rat livers (results not shown). For determination of the expression profiles of HATs during hepatocarcinogenesis, we performed Western blot analysis using nuclear extracts and specific antibodies to each of the HATs (Figure 1B). The HATs best characterized as transcriptional co-activators are p300, CBP, P/CAF and GCN5. The expression levels of P/CAF and GCN5 showed no change during hepatocarcinogenesis, but expression of both p300 and CBP decreased. Next, we observed the expression levels of the MYST-type acetyltransferases, which are involved in a wide range of regulatory functions [1]. Expression of TIP60 was unchanged during hepatocarcinogenesis, whereas MOZ expression increased. MORF was not detected (results not shown). Among those we assayed, MOZ was the sole HAT whose expression was positively correlated with GSTP expression during hepatocarcinogenesis.

Induction of the intact form of MOZ during hepatocarcinogenesis

MOZ belongs to the MYST family of HATs and frequently is rearranged in leukaemia [10]. MOZ fusion partners include CBP, p300 and TIF2 (transcriptional intermediary factor 2); all of these proteins are also known to be transcriptional co-activators [10,24–26]. MOZ is a transcriptional regulator in haemopoiesis, and MOZ fusion proteins antagonize MOZ function and lead to leukaemogenesis [26,28]. Using Western blotting and RT (reverse transcriptase)–PCR analyses, we assessed whether MOZ was translocated and thus fused with these transcriptional co-activators during hepatocarcinogenesis (Figure 2 and results not shown). We generated specific antibodies against the N- and C-terminal regions of rat MOZ, and we also used the anti-MYST antibody, which recognizes a motif (amino acids 671–685) in the MYST region of MOZ. These three antibodies recognize different parts of MOZ. Western blot analysis revealed that MOZ induced in livers with hyperplastic nodules and recognized by the three different antibodies were all the same size (Figure 2A), as

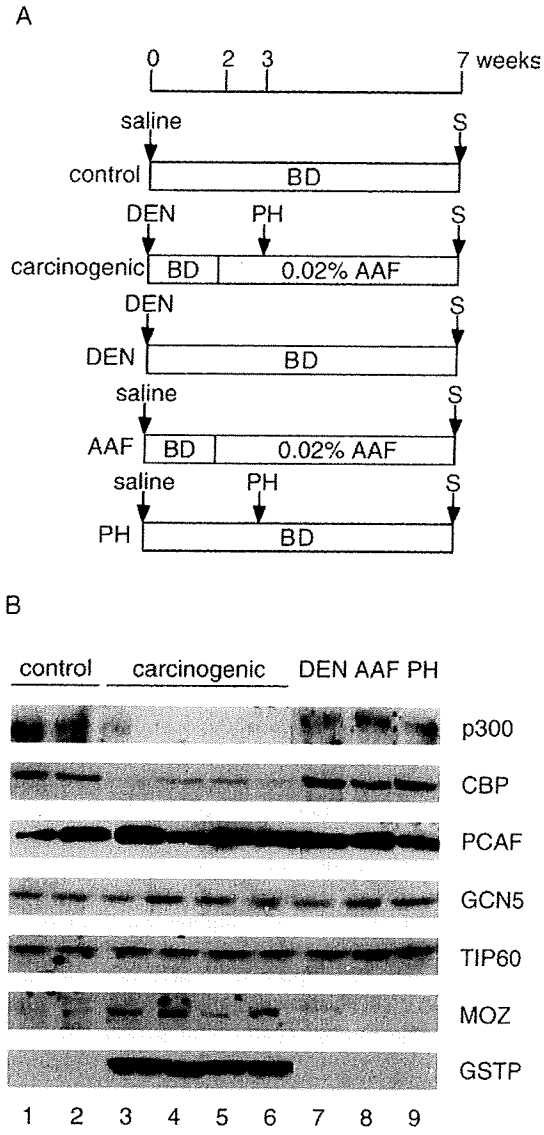

Figure 1 Expression profiles of HAT during hepatocarcinogenesis

(**A**) The Solt–Farber protocol for chemically induced hepatocarcinogenesis in rats [37]. BD, basal diet; S, times at which rats were killed. (**B**) Expression profiles of HATs were investigated in control livers and those with hyperplastic nodules. Nuclear extracts were prepared from livers of various rats, and immunoblot analysis was performed with specific antibodies, as described in the Experimental section. GSTP in the cytosol fraction was also detected (bottom). The fractions shown in lanes 1 and 2 were from control rats; lanes 3–6, rats having livers with hyperplastic nodules; lane 7, rat treated with DEN only; lane 8, rat treated with AAF only; lane 9, rat underwent PH only.

was the less-abundant MOZ in the control rat liver. We characterized additional fusion partners, including p300, CBP and TIF2. The sizes of these proteins in livers with hyperplastic nodules were the same as those in control livers (Figures 1B and 2B). These results suggested that the intact form of MOZ was induced and that translocation of MOZ did not occur during chemical hepatocarcinogenesis in rats. To confirm these results, we performed RT–PCR with three sets of primers spanning the MOZ regions in which rearrangement occurred frequently [10,24–26]. Sequencing of PCR products revealed that MOZ rearrangement did not occur during hepatocarcinogenesis (results not shown). We further examined the MOZ–CBP chimaeric transcript by hemi-nested PCR, but the amplification product was not detected

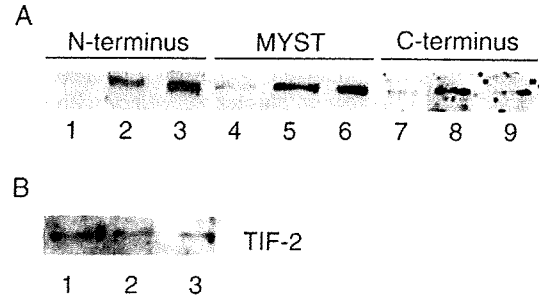

Figure 2 Induction of the intact form of MOZ during hepatocarcinogenesis

(**A**) Nuclear extracts were prepared from control (lanes 1, 4 and 7) and livers with hyperplastic nodules (lanes 2, 3, 5, 6, 8 and 9), separated by SDS/PAGE (7.5% gel), and immunoblotted using polyclonal antibodies against the N- (lanes 1–3) or C- (lanes 7–9) terminal region of MOZ or the anti-MYST antibody (lanes 4–6). (**B**) The putative MOZ fusion partner, TIF-2, was detected with the anti-TIF-2 antibody. The fraction shown in lane 1 is from control; those in lanes 2 and 3 were from livers with hyperplastic.

(results not shown). These results indicate that the intact form of MOZ was induced and that MOZ translocation did not occur during the early stages of hepatocarcinogenesis.

Activation of GSTP promoter activity by MOZ through the GPE

MOZ functions as a transcriptional co-activator and participates as a mediator in haemopoiesis [28,42]. To characterize the effect of MOZ on hepatocarcinogenesis-specific gene expression, we asked whether exogenous MOZ would enhance GSTP promoter activity. GSTP is strongly and specifically expressed during chemical hepatocarcinogenesis and is considered to be an excellent tumour marker [29]. The transcriptional regulatory region of the rat GSTP gene includes enhancer and silencer elements [30,31]. To examine the effect of MOZ on GSTP promoter activity, −2.5GST-luciferase (which has the entire GSTP regulatory region and promoter) was co-transfected with MOZ expression plasmid or control empty vector into rat hepatoma H4IIE cells (Figure 3A). MOZ enhanced GSTP promoter activity (Figure 3B). Luciferase activity in the presence of various concentrations of MOZ was assayed, and MOZ demonstrated dose-dependent enhancement of GSTP promoter activity (Figure 3C). To more closely define the MOZ response element, we used two reporter plasmids: −2.15GST-luciferase, which lacked the GPE, and −91GST-luciferase, which lacked both the GPE and silencer regions (Figures 3A and 3B). These reporter plasmids were not transactivated, thereby suggesting that MOZ activates GSTP promoter activity through the GPE.

MOZ interacts with MafK both *in vitro* and *in vivo*

The GPE1 element in GPE is a key control element responsible for GSTP expression in preneoplastic tissue. GPE1 is similar in sequence to ARE (antioxidant-response-like element), MARE (Maf recognition element) and TRE [PMA ('TPA')-responsive element] [30,33,34]. A recent study showed that the Nrf2–MafK heterodimer binds to GPE1 and regulates GSTP promoter activity [34]. To determine the mechanism of the MOZ-associated enhancement of GSTP promoter activity, we tested whether MOZ could bind Nrf2 and MafK. We previously showed that MOZ interacted with c-Jun through the bZIP domain *in vitro* [39]; Nrf2 and MafK also have bZIP domains. To determine MOZ binding partners, we performed an *in vitro* pull-down assay using ^{35}S-labelled full-length MOZ. We fused the Nrf2 DNA-binding domain to maltose-binding protein, incubated it with ^{35}S-labelled MOZ, and precipitated it with amylose resin, but interaction

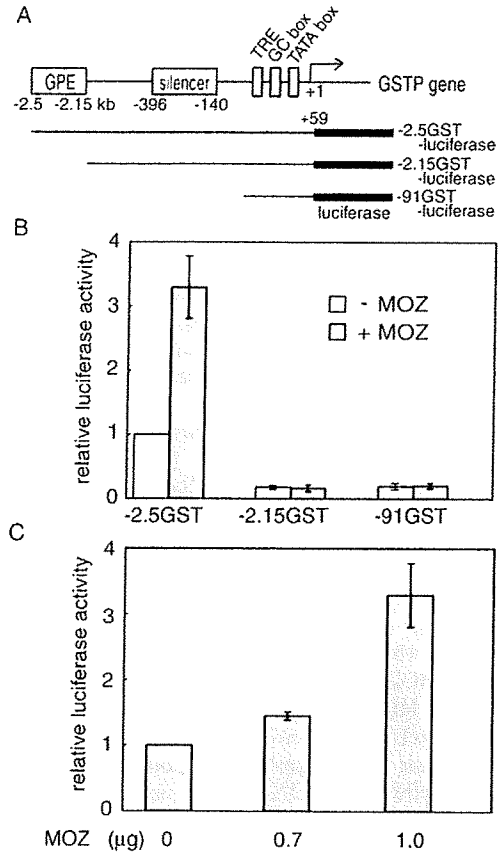

Figure 3 MOZ activates the GSTP promoter activity through the GPE

(**A**) Diagram of the 5′-flanking region of the rat GSTP gene and the reporter constructs for observing the effect of MOZ on the promoter activity of the GSTP gene. (**B**) We co-transfected 100 ng of the reporter plasmid with (grey columns) or without (white columns) 1 μg of MOZ expression plasmid (pCI-MOZ) into H4IIE rat hepatoma cells. All transfection assays were repeated at least three times. The relative luciferase activity was calculated from mean values relative to the activity of −2.5GST-luciferase in the absence of MOZ. Each error bar indicates ±S.D. (**C**) Dose-dependent transactivation of −2.5GST-luciferase by MOZ. Relative luciferase activities are shown as in (**B**).

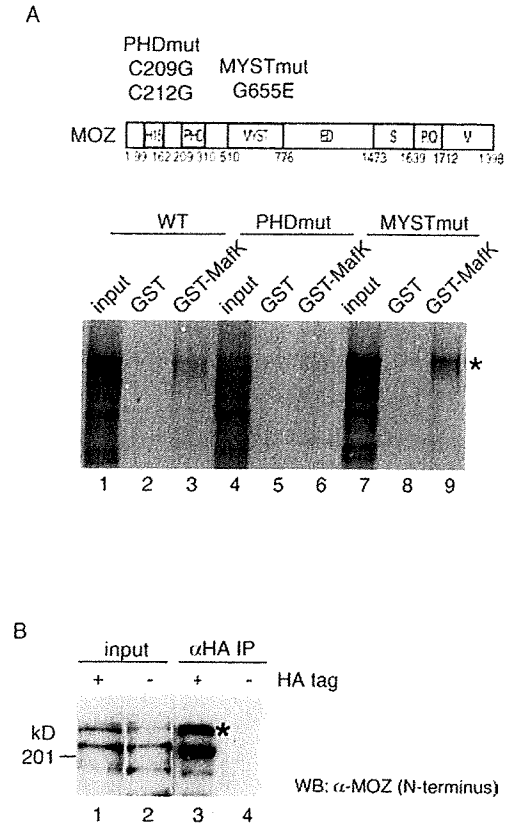

Figure 4 MOZ interacts with MafK *in vitro* and *in vivo*

(**A**) Structural domains of MOZ were indicated as follows: H15, histones H1- and H5-like module; MYST, MYST acetyltransferase domain; ED, glutamic acid/aspartic acid-rich acidic regions; S, serine-rich domain; P/Q, proline/glutamine-stretch; and M, methionine-rich domain. Also shown are the mutation positions in the PHD finger and MYST regions. Indicated wild-type and mutated *in vitro*-translated [^{35}S]MOZ proteins were incubated with GST (lanes 2, 5 and 8) or GST–MafK (lanes 3, 6 and 9). MOZ protein retained on the GST-conjugated beads after extensive washing was analysed by SDS/PAGE and autoradiography. The amount of input (lanes 1, 4 and 7) is equivalent to 10% of the reaction volume in the assay. [^{35}S]MOZ proteins are indicated by asterisks (*). (**B**) MOZ expression plasmid was co-transfected with HA-tagged MafK (lanes 1 and 3) or non-tagged MafK (lanes 2 and 4) into HeLa cells, and nuclear extracts were prepared. Immunoprecipitation (IP) experiments were performed with anti-HA antibody. Immunoprecipitates (lanes 3 and 4) and 5% of input (lanes 1 and 2) were resolved by SDS/PAGE (7.5% gel) and detected by Western blotting using anti-N-terminal MOZ antibody. MOZ proteins are indicated by asterisks (*).

between MOZ and the Nrf2 DNA-binding domain was not detected (results not shown). Next, we evaluated the interaction between MOZ and MafK using a GST pull-down assay and found that ^{35}S-labelled MOZ interacted with GST–MafK but not with GST alone (Figure 4A, lanes 1–3). Unique structural domains are identified in MOZ [28]. To identify the region required for the interaction between MOZ and MafK, two MOZ derivatives with double and single point mutations in the PHD zinc-finger (C209G and C212G) and the MYST (G655E) regions respectively, were generated. The PHD zinc-finger and the MYST regions are important for binding to specific nuclear protein partners and HAT activity, respectively [43,44]. The mutant in the PHD zinc-finger region was not able to interact with GST–MafK, whereas the mutation in the putative acetyl-CoA-binding site in the MYST region did not affect the binding to MafK (Figure 4A, lanes 4–9). These results suggest that MOZ interacts with MafK in the absence of the heterodimer partner, Nrf2, mediated by the PHD zinc-finger region of MOZ.

To evaluate the interaction between MOZ and MafK under physiological conditions, we attempted to detect immunoprecipitated MOZ, but endogenous MOZ in nuclear extracts from H4IIE and HeLa cells could not be detected. Therefore we next introduced the MOZ expression plasmid with HA-tagged or non-tagged MafK into HeLa cells, and nuclear extracts were prepared. MOZ was immunoprecipitated only in nuclear extracts expressing HA-tagged MafK (Figure 4B). Some degraded MOZ proteins were detected in nuclear extracts and these proteins were also immunoprecipitated. GST pull-down and immunoprecipitation experiments suggest that MOZ may interact with the MafK moiety of the Nrf2–MafK heterodimer *in vivo*.

MOZ functions as a co-activator of the Nrf2–MafK heterodimer

MOZ preferentially interacted with MafK and up-regulated GSTP promoter activity through GPE, which contains the binding site for the Nrf2–MafK in the reporter assay (Figures 3 and 4). These data suggest that MOZ is a potential co-activator of Nrf2–MafK heterodimer. To test this hypothesis, we investigated whether MOZ could stimulate Nrf2–MafK-mediated transactivation (Figure 5). We have previously reported that Nrf2 simulates GPE1-mediated transactivation in F9 cells, which are considered to lack AP1 (activator protein 1) activity and to express excess amounts of small Maf proteins, including MafK [34]. MOZ or Nrf2